Mechanism of DNA Chain Initiation by the dnaG Protein of Escherichia coli

by

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B.S., Massachusetts Institute of Technology
(1976)

SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE
DEGREE OF

DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
January 1981

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Submitted to the Department of Biology on January 28, 1981
in partial fulfillment of the requirements for the degree
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ABSTRACT

All known DNA polymerases are unable to initiate the synthesis of DNA chains de novo, but are capable of extending the 3' hydroxy terminus of a preexisting 'primer' chain stably annealed to the template strand. The report that partially purified preparations of the dnaG protein, a gene product essential to the replication of E. coli, synthesize RNA primers on phage G4 single-stranded DNA (Bouche, Zechel and Kornberg, 1975) stimulated an investigation into the properties of this enzyme.

A thermolabile dnaG protein, prepared from a temperature-sensitive strain of E. coli, was utilized to demonstrate that the ability to prime DNA synthesis on phage G4 and øx174 single-stranded DNA resides with the dnaG protein, and that the priming event may be separated from subsequent DNA synthesis. Priming on G4 DNA absolutely requires the E. coli DNA binding protein. In contrast, øx174 DNA must first be 'activated' in a reaction requiring several other proteins and ATP (Ray, Capon and Gefter, 1976). dNTPs as well as rNTPs serve as substrates for priming on G4 DNA and appear to be essential for priming on øx174 DNA.

The dnaG protein initiates the synthesis of the complementary strand of phage St-1, øK and α3 as well as G4 single-stranded DNA by synthesizing a specific oligonucleotide primer. The mechanism of this reaction in the absence of DNA synthesis was investigated. rNTPs and dNTPs serve as substrates in this reaction and hybrid primers are synthesized when both are present together. The oligoribonucleotide primers synthesized on St-1, øK and α3 DNA are each unique and have an identical nucleotide sequence. Primer synthesis begins at a unique site on each template with ATP or dATP, and extends for 10 to 28 nucleotides. The length of the products is affected by the concentration of substrates, as well as salt concentration and temperature in accordance with their effect on secondary structure. The longest products copy a template hairpin sequence consisting of 8 contiguous base-pairs that begins 6 nucleotides downstream from the start site.

dNTPs are incorporated into the primer synthesized on St-1 DNA at every position occupied by the homologous rNTP. When present at equal concentrations, dNTPs and rNTPs are incorporated into hybrid primers with the same efficiency, although ATP is preferred to dATP as the initiating

nucleotide. There appears to be no preference for rNTP or dNTP selection with respect to the position in the primer sequence, except for that noted, or with respect to the composition of the preceding residue.

When primer formation is coupled to DNA synthesis catalyzed by either DNA polymerase III with the dnaZ protein and elongation factors I and III or DNA polymerase I, primer synthesis terminates earlier. However, greater than 90% of the DNA product is still initiated from primers at least 9-11 nucleotides in length. The coupling effect observed is the same with either DNA polymerase.

Thesis advisor: Dr. Malcolm L. Gefter
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4 MAP p.5

Dedication

in memory of my loving and beloved brother,

Howard Alan Capon

and to my parents, grandparents, brother Robert,

and last but not least,

my wife Ronna

TABLE OF CONTENTS

Page

List of Figures Page

Page

List of Tables

Introduction

The initiation of DNA replication is currently appreciated as an elaborate process involving many proteins which may comprise a weakly held together 'replication complex'. The purpose of this discussion will be to point out those considerations which have led to this understanding and to review the studies concerning this process which have been conducted on the bacterium Escherichia coli. This organism has been studied most extensively, largely because of the ability to obtain genetic lesions which interrupt the process, and the availability of a variety of phage and plasmid DNA's for biochemical analysis which rely upon the host apparatus for their own replication.

DNA synthesis is accomplished by the enzyme DNA polymerase which catalyzes the stepwise polymerization of nucleotides into a polynucleotide chain with a sequence complementary to that of the parental DNA template strand. The immediate nucleotide precursors for the polymerization event are 5' deoxyribonucleoside triphoshates. One functional role that has been ascribed to several of the protein factors involved in the initiation of DNA replication was suggested by the inability of all template-directed DNA polymerases studied to initiate DNA synthesis de novo. Unlike RNA polymerizing enzymes which are capable of initiating synthesis at specific nucleotide sequences on the template strand, DNA polymerase may only covalently extend the pre-existing 3' OH terminus of a polynucleotide 'primer' chain that is stably base-paired to the template. Primers comprised of ribonucleotides, as well as deoxyribonucleotides, promote in vitro DNA synthesis by all DNA polymerases.

Several mechanisms for the generation of primers involving distinct replication proteins have recently been identified and will be considered below in the context of type of initiation event they promote.

As suggested above the process of primer formation determines the location on the DNA template at which DNA synthesis begins. DNA replication has been shown to begin at a unique chromosomal site for many bacteria, phages and plasmids (Kornberg, 1979). The E. coli origin was mapped near the ilv locus by comparing the relative amounts of bacteriophage Mu and λ DNA in exponentially growing cultures of cells which had λ integrated at its normal attachment site and Mu integrated at different positions on the E. coli chromosome (Bird et al., 1972). Since the relative amount of Mu-hybridizing DNA increased as the phage was integrated closer to the ilv locus from either side, this study also demonstated that replication in E. coli is bidirectional; that is, a replication fork proceeds outward in each direction from this origin.

Recently, self-replicating plasmids containing the E. coli origin as their only origin have been isolated by in vitro cloning (Yasuda and Hirota, 1977) and by transduction with phage λ (von Meyerberg et al., 1978). Selective deletion of DNA has led to the identification a 300 nucleotide long region essential to initiation of DNA replication. The nucleotide sequence of this region, as well as those for phages λ, G4, St-1, α3, φK, and fd, and the plasmid ColE1, exhibits a high degree of potential secondary structure generally not observed in structural gene sequences (Sugimoto et al., 1978, Meijer et al., 1978, Hobom et al., 1978, Sims and Dressler, 1978, Fiddes, Barrell and Godson, 1978, Sims, Capon and Dressler, 1979, Schaller, Beck and Takanami, 1978, Suggs and Ray, 1978, and Backman et al.,

1978). Thus, it would not be surprising if replication proteins were responsible for the specificity observed in selecting the chromosomal initiation site, although the nature of such interactions has only begun to be examined in greater detail.

The need for a distinct type of initiation event was suggested by the demonstration of the discontinuous nature of replicative DNA synthesis in E. coli (Okazaki et al., 1968). All DNA polymerases studied thus far catalyze deoxyribonucleotide addition in the 5' → 3' direction exclusively. Propagation of the replication fork requires that as the parental DNA strands are unwound, each will direct the synthesis of a complementary daughter strand (Meselson and Stahl, 1958). A mechanism was sought, therefore, for the synthesis of the daughter strand that proceeds in the 3' → 5' direction (lagging strand).

It was demonstrated that when a short pulse of (^{3}H) thymidine was delivered to exponentially growing cultures of E. coli much of the radioactivity was first incorporated into slowly sedimenting DNA chains (10S) 1000-2000 nucleotides in length (Okazaki et al., 1968). This experiment suggested that each newly made strand was constructed from shorter 10S chains which had been synthesized in the 5' → 3' direction by DNA polymerase once a sufficient length of the template strands was exposed by unwinding, and subsequently joined together by polynucleotide ligase. Consistent with this hypothesis was the observation that ligase-deficient mutants of E. coli accumulated 10S DNA fragments (Konrad, Modrich and Lehman, 1973, Gottesman, Hicks and Gellert, 1973). Thus, as the replication fork progresses repeated initiation of short DNA chains, or 'Okazaki pieces', is required.

The relevance of Okazaki pieces as E. coli DNA replication intermediates has been recently challenged on several grounds. First, in order to demonstrate a precursor-product relationship it is necessary to follow the fate of 10S DNA chains which have been labeled briefly. The large size of the precursor pool makes it difficult, however, to introduce radioactive precursor for short time periods without resorting to severe conditions such as thymine starvation. This introduces new difficulties since the number and size of Okazaki pieces observed depends dramatically upon the conditions used to deliver the pulse (Werner, 1971, Diaz, Wiener and Werner, 1975, Brewin, 1977). Recently, by examining the disappearance of short DNA chains when cells grown in radioactive media for several generations are transferred to unlabeled media it was demonstrated that most of the 20-40 short DNA pieces (700-9000 nucleotides in length) that occur per cell in the steady-state do not behave as expected for true DNA replication intermediates (Anderson, 1978).

One possible origin of the non-replicative class of Okazaki pieces is the strand-nicking due to excision-repair of dUMP residues which have been misincorporated into DNA in place of dTMP. This was suggested by experiments which show that sof mutants of E. coli, which accumulate very small DNA chains (4-6S) when pulsed with (^{3}H) thymidine, are deficient in the enzyme dUTP hydrolase (Tye et al., 1977). The increase in cellular dUTP levels in this mutant leads to more frequent transient misincorporation of dUMP. However, although ung mutations, which cause deficiencies in the enzyme responsible for initiating the dUMP excision-repair pathway (uracil N-glycosidase), may supress the accumulation of 4-6S DNA chains in sof mutants, the ung mutation does not reduce the level of 10S Okazaki

pieces found in an otherwise normal strain (Lehman, Tye and Nyman, 1978). Thus, although the excision-repair of dUMP is not the only mechanism by which Okazaki pieces arise, the contribution of other repair processes has not been ruled out.

These observations are particularly interesting in light of the uncertainty concerning whether one or both of the newly replicated strands are synthesized discontinuously. On the basis of annealing experiments it was shown that two classes of short DNA chains synthesized in an *in vitro* system, approximately 10S and 38S in size, correspond to synthesis directed by opposite parental strands (Hermann, Huf and Bonhoeffer, 1972). Similarly, most of the pulsed-labelled 10S DNA synthesized *in vivo* by a strain of *E. coli* lysogenic for phage λ hybridizes to one strand of the phage DNA, whereas most of the pulsed-labelled DNA hybridizing to the other strand is longer (approximately 35S). Knowledge of the orientation of the integrated phage DNA demonstrates that the 10S DNA pieces represent synthesis of the lagging strand (Louarn and Bird, 1974). However, it remains uncertain whether synthesis of the longer 'leading strand' (the strand synthesized in the overall direction of replication fork movement) represents discontinuous synthesis that is less frequently initiated than that of the lagging strand, or rather, continuous synthesis which appears to be interrupted due to repair-related nicking.

Initiation at the chromosomal origin of DNA replication and the initiation of DNA chain synthesis in *E. coli* are distinguished on the basis of susceptibility to rifampicin and chloramphenicol. The initiation of new rounds of chromosomal replication is inhibited by both drugs (suggesting a requirement for protein synthesis), whereas completion of ongoing

rounds, which presumably requires repeated initiation of many new DNA chains, is unaffected. Two lines of evidence suggest that transcription is also required for chromosomal initiation. First, the process is not inhibited by rifampicin in strains which have a rifampicin-resistant RNA polymerase. Secondly, just prior to initiation, when chloramphenicol is no longer inhibitory, rifampicin will still block initiation (Lark, 1972a). However, it is not yet understood whether the product of this transcriptional event serves as a primer for DNA synthesis at the origin or 'activates' the origin for initiation in some other fashion.

Studies with E. coli mutants bearing temperature-sensitive defects in DNA synthesis has revealed additional differences between chromosomal initiation and the initiation of DNA chains. Chromosomal initiation mutants are those which complete ongoing rounds of DNA replication after a shift to the temperature which is non-permissive for growth, but do not begin new rounds. This category includes dnaA, dnaI and dnaP mutants as well as certain alleles of the dnaB and dnaC genes (Hirota, Ryter and Jacob, 1968, Beyersmann, Messer and Schilcht, 1974, Wada and Yura, 1974, Zyskind and Smith, 1977, Schubach, Whitmer and Davern, 1973). Mutants which immediately cease all DNA synthesis when shifted to the restrictive temperature include both defects in DNA chain elongation, such as dnaE- DNA polymerase III (Gefter et al., 1972) and dnaZ- elongation factor II (Wickner and Hurwitz, 1976), and defects in DNA chain initiation. The latter group includes the dnaG, dnaB and dnaC genes (Carl, 1970, Wechsler and Gross, 1971). The product of the dnaG gene has been implicated in the initiation of Okazaki pieces both in vitro (Lark, 1972b) and in vivo

(Louarn, 1974). It is pointed out that the criteria used to classify an immediate-stop mutation does not rule out a role for the same gene product in chromosomal initiation, which is consistant with the observation that both dnaB and dnaC (and possibly dnaG) may contribute to each process.

Common to each event is the requirement for the generation of a primer for DNA synthesis. The mechanism of primer formation during the initiation of DNA chain synthesis has been illuminated by studies on the single-stranded DNA phages of E. coli. The first stage in the overall replication of these phages is the conversion of the circular single-stranded viral DNA to the double-stranded replicative form, a process which is not inhibited by chloramphenicol, and therefore, depends entirely upon the host replication apparatus. Whereas conversion of infecting phage M13 DNA is inhibited by rifampicin, suggesting that RNA polymerase synthesizes the M13 primer (Brutlag, Schekman and Kornberg, 1971), the conversion of infecting ϕx174 DNA is not affected.

These reactions have been extensively studied in an in vitro system consisting of a soluble extract prepared from gently lysed cells which replicates exogenously added M13 (or fd) and ϕx174 single-stranded DNA. Although as in vivo, rifampicin selectively inhibits M13 and not ϕx174 replicative form synthesis in vitro, evidence was obtained for the covalent attachment of ribonucleotides to the 5' end of the nascent DNA product in each case (W. Wickner et al., 1972, Schekman et al., 1972). As discussed below, this led to the discovery of a rifampicin-resistant priming mechanism which employs the product of the dnaG gene (Bouche, Zechel and Kornberg, 1975).

Much evidence has accumulated to suggest that the initiation of DNA chain synthesis in E. coli is similar to the initiation of øx174 replicative form synthesis. Both utilize a rifampicin-resistant mechanism of priming and require functional dnaB, dnaC and dnaG, but not dnaA gene products (R. Wickner et al., 1972, Schekman et al., 1972, Wickner, Wright and Hurwitz, 1973). In each case priming is probably random with respect to the nucleotide sequence of the template strands (Eisenberg et al., 1975, McMacken, Ueda and Kornberg, 1977), although one report has suggested that E. coli Okazaki fragments synthesized in vitro may not be initiated at random with respect to 6-methyl adenine residues which occur on the template, as determined by their lack of susceptibility to cleavage with endonuclease Dpn I (Gomez-Eichelmann and Lark, 1977). Thus, it has been the expectation that E. coli nascent DNA chains are also initiated with RNA.

There has been much controversy concerning the covalent attachment of RNA to the 5' end of nascent DNA chains in E. coli (see Gefter, 1975, Ogawa and Okazaki, 1980). The original reports which demonstrated a buoyant-density shift (in Cs_2SO_4 gradients) of E. coli Okazaki pieces upon treatment with alkali or ribonuclease (Sugino, Hirose and Okazaki, 1972, Hirose, Okazaki and Tamanoi, 1973) have been attributed to the non-specific aggregation of RNA and DNA in Cs_2SO_4 gradients rather than covalent linkage (Gefter, 1975, Ogawa et al., 1977). Other evidence has been provided by the following approaches: 1) transfer of (^{32}P) from deoxyribonucleotides to neighboring ribonucleotides upon alkali treatment of nascent E. coli DNA chains synthesized in vitro with (α-^{32}P) dNTP's in either toluene-permeabilized cells (Sugino and Okazaki, 1973, Okazaki et al., 1975) or cell lysates (Ramareddy et al., 1975), 2) detection of 5' OH termini of in vivo nascent E. coli

DNA chains after alkaline hydrolysis by either susceptibility to degradation by calf spleen phosphodiesterase (Kurosawa et al., 1975) or phosphorylation with radioactive phosphate from (γ-^{32}P) ATP by phage T4 polynucleotide kinase (Ogawa et al., 1977). However, there are possible drawbacks to each of these methods. For example, the phosphate-transfer method will also detect RNA-DNA linkages that arise from the misincorporation of ribonucleotides into DNA by DNA polymerase I (Berg, Fancher and Chamberlain, 1963) or from the incorporation of ribonucleotides onto DNA by RNA polymerase (S. Wickner et al., 1972). In addition, those methods which detect 5' OH termini of DNA after alkali treatment may also detect apyrimidinic sites on the DNA that arise from excision of misincorporated dUMP residues by uracil N-glyclosidase.

Two groups have reported that RNA is not found at the 5' end of nascent DNA chains in E. coli (Uyemura, Eichler and Lehman, 1976, Denhardt, Kowalski and Miyamato, 1978). There has been some dispute in this regard as to what is actually considered a nascent DNA replication intermediate in E. coli (Ogawa and Okazaki, 1980). However, there are several reports concerning other procaryotic and eucaryotic systems in which the covalent attachment of RNA to the 5' end of DNA replication intermediates is well established employing the same criteria described above (see DePamphilis and Wasserman, 1980). Perhaps the most convincing evidence for the detection of RNA-linked nascent DNA pieces in E. coli concerns phage T4 and T7 replication intermediates in vivo from which the actual ribonucleotide primer molecule has been isolated by digestion with the 3' → 5' exonuclease activity of T4 DNA polymerase and shown to correspond to the primer synthesized in vitro (Okazaki et al., 1978, Kurosawa and Okazaki,

1979, Seki and Okazaki, 1979). The T_7 and T_4 priming mechanisms, which employ phage-encoded priming proteins, will be considered below.

The initiation of DNA chain synthesis in E. coli most likely occurs on a region of single-stranded DNA template at the replication fork. Three pathways for the initiation of DNA synthesis on single-stranded DNA have been established by in vitro studies on the single-stranded DNA phages of E. coli (Schekman, Weiner and Kornberg, 1974, Wickner and Hurwitz, 1974, Wickner, 1980). The reactions which convert single-stranded fd (or M13), G4 and øx174 DNA to the double-stranded replicative form have been reproduced with extensively purified proteins. Two strategies have been utilized to identify the appropriate factors: 1) in vitro complementation assay- Cell extracts which normally support phage DNA synthesis in vitro may be deleted specifically for a single required protein by preparing the extract from a thermosensitive dna mutant strain. The deleted activity may be identified during purification from a wild-type cell extract by its ability to restore DNA synthesis to the thermosensitive extract. To conclusively prove the identity of a protein thus determined it is necessary to purify the activity from the corresponding dna^{ts} strain and demonstrate that the protein is thermolabile; 2) In most instances the replication proteins purified from existing thermosensitive strains have proven insufficient to catalyze the overall reaction of interest alone, but have served as the basis for the identification of the remaining factors in crude extracts. This approach poses two drawbacks. First, it has resulted in certain cases in the reconstitution of a system which is not specific for the template under study. In addition, it is often difficult to establish the relation between proteins purified on

this basis alone and those factors which are required for in vivo DNA replication.

For each of the three pathways mentioned above, to observe initiation which is specific for a particular DNA template it is necessary to include the single-stranded DNA binding protein (helix-destabilizing protein) of E. coli in the reaction. This protein binds tightly and cooperatively to single-stranded, but not double-stranded, DNA and thereby is able to enhance the denaturation of duplex DNA (Sigal et al., 1972). In addition, the DNA binding protein is an essential component of the ATP-dependent unidirectional DNA unwinding reactions catalyzed by the E. coli rep protein in the 5' → 3' direction (Yarranton and Gefter, 1972, Scott et al., 1977) and by E. coli helicase III in the 3' → 5' direction (Yarranton, Das and Gefter, 1979). These studies show that the DNA binding protein functions by preventing strand renaturation, and thus suggests a central role for the protein in maintaining the integrity of the replication fork. Consequently, it is likely that the natural template for the initiation of DNA chain synthesis in E. coli is DNA covered with the DNA binding protein.

The DNA binding protein also binds specifically to E. coli exonuclease I and DNA polymerase II, and to T7 DNA polymerase, and forms a ternary complex with DNA and either of the two latter enzymes (Molineaux and Gefter, 1974, 1975). When DNA polymerase II is complexed in this fashion the enzyme no longer dissociates from the template at regions of secondary structure and thereby catalyzes DNA synthesis in a processive manner (Sherman and Gefter, 1976). Thus, the DNA binding protein may contribute to the conversion of the single-stranded phage DNA's to replicative form

by either facilitating the template-specific (or site-specific) binding of the initiation proteins to the DNA, or in addition, by modifying the catalytic activity of the priming protein(s).

The three pathways described below are distinguished by their mechanism of primer formation rather than DNA chain elongation (Wickner and Hurwitz, 1974, Wickner, 1976, Schekman, Weiner and Kornberg, 1974). Given a primer all of the single-stranded phage DNA templates studied appear to utilize equally well either DNA polymerase I, II or III in conjunction with the appropriate set of elongation factors (Wickner, 1980).

Priming of phage fd or M13 DNA synthesis requires E. coli RNA polymerase, DNA binding protein and all four ribonucleoside triphosphates (Geider and Kornberg, 1974). The origin of complementary strand DNA synthesis lies in an intercistronic region of approximately 500 nucleotides which exhibits an extensive capacity for secondary structure and which is highly conserved (particularly the secondary structure) among the related phages fd, M13 and f1 (Schaller, Beck and Takanami, 1978, Suggs and Ray, 1978, Horiuchi, Ravetch and Zinder, 1978). In the presence of the DNA binding protein RNA polymerase catalyzes the synthesis of a specific ribonucleotide primer for DNA synthesis that is complementary to the DNA template at the origin (Geider, Beck and Schaller, 1978). Primer synthesis begins at a unique site several nucleotides upstream from one of the hairpin structures, is initiated only with ATP, and extends for approximately 30 nucleotides. Synthesis is terminated near the top of the hairpin; it has been suggested that the opposite strand of the hairpin becomes complexed with DNA binding protein as it is unwound during transcription and that this results in termination of primer synthesis at one of several positions

(Geider, Beck and Schaller, 1978).

A segment of single-stranded fd DNA, approximately 125 nucleotides in length, which contains two hairpin structures including the one which is transcribed, is protected from nuclease digestion in the presence of RNA polymerase and DNA binding protein (Schaller, Uhlmann and Geider, 1976). As previously proposed, this is consistent with a role for the DNA binding protein in directing RNA polymerase to a specific initiation site on the template (Geider and Kornberg, 1974). Despite this apparent template specificity, øx174 replicative form synthesis is primed equally well by RNA polymerase, DNA binding protein and ribonucleotides (Wickner and Kornberg, 1974, Vicuna et al., 1977). As mentioned earlier, however, øx174 DNA synthesis catalyzed by crude extracts requires the products of the dnaG, dnaB and dnaC genes even in the presence of a functional RNA polymerase. On this basis one group has purified those additional factors present in the crude extract which make priming by RNA polymerase specific for fd, and not øx174, DNA (Vicuna et al.,1977, Vicuna, Ikeda and Hurwitz, 1977). One of these factors has been identified as E. coli ribonuclease H, which presumably may act to degrade non-specific ribonucleotide primers, whereas the function of the other two components, discriminatory factors α and β, is not yet understood.

As opposed to the filamentous phages of E. coli such as fd which depends upon RNA polymerase to synthesize its primer, all of the isometric single-stranded DNA phages appear to be primed by the action of the dnaG gene product. Two distinct mechanisms have thus far been identified, the simpler of which is typified by phages G4 and St-1, and requires only DNA binding protein in addition to the dnaG protein (Zechel, Bouche and

Kornberg, 1975). At least five additional proteins are required for dnaG catalyzed priming of øx174 replicative form synthesis (Wickner and Hurwitz, 1974, Schekman, Weiner and Kornberg, 1974).

The dnaG protein was originally purified by its ability to complement extracts prepared from dnaG^{ts} strains for øx174 replicative form synthesis (Wickner, Wright and Hurwitz, 1973) or E. coli DNA synthesis on cellophane discs (Nusslein et al., 1973). The protein consists of a single polypeptide subunit with a native molecular weight of 60,000 daltons that binds weakly to denatured DNA (Wickner, Wright and Hurwitz, 1973). In a rifampicin-resistant reaction containing G4 DNA, DNA binding protein and ribonucleoside triphosphates the dnaG protein catalyzes the synthesis of a specific oligoribonucleotide primer for G4 replicative form synthesis that becomes covalently attached to the DNA product synthesized by DNA polymerase III and elongation factors (Bouche, Zechel and Kornberg, 1975). Studies with a thermolabile protein prepared from a dnaG^{ts} strain have confirmed that the priming and oligonucleotide-synthesizing activities reside with the dnaG protein (Wickner, 1977, Capon and Gefter, 1978). In the presence of the DNA binding protein the dnaG protein catalyzes priming with, and binds specifically to, G4 or St-1, but not øx174 or fd, DNA (Wickner, 1977).

The dnaG protein may also utilize deoxyribonucleoside triphosphates as substrates for the synthesis of primers with G4 or St-1 DNA that are comprised of both ribo- and deoxyribonucleotides (Wickner, 1977, McMacken et al., 1977, Capon and Gefter, 1978). Two groups have reported that deoxyribonucleotides are incorporated less efficiently than ribonucleotides and reduce the overall rate of oligonucleotide synthesis in reactions

containing both types of substrates (Rowen and Kornberg, 1978, Benz et al., 1980). In contrast, another study has shown that dGTP is utilized at least as efficiently as GTP for oligonucleotide synthesis with G4 DNA Wickner, 1977). It has been reported that the dnaG protein may initiate G4 primer synthesis with ATP, but not dATP, and that although a 29 nucleotide long primer is made in the absence of DNA synthesis, DNA polymerase may displace the dnaG protein and covalently extend a primer as short as a dinucleotide (Rowen and Kornberg, 1978).

The nucleotide sequence of the unique oligoribonucleotide synthesized by the dnaG protein with G4 DNA, which is 26 to 29 nucleotides in length, is complementary to the DNA template at the site at which replicative form synthesis is initiated in vivo (Bouche, Rowen and Kornberg, 1978, Hourcade and Dressler, 1978, Sims and Dressler, 1978, Fiddes, Barrell and Godson, 1978). The origin of G4 complementary strand synthesis lies in an intercistronic region of 135 nucleotides which is highly conserved in the related phages St-1, α3 and øK, all of which are primed by the dnaG protein and DNA binding protein (Sims, Capon and Dressler, 1978, D. Capon and M. Gefter, unpublished observations).

In addition to the dnaG protein and DNA binding protein initiation of øx174 replicative form synthesis requires the products of the dnaB and dnaC genes and at least three other protein factors: replication factors X, Y and Z (Wickner and Hurwitz, 1974) or factors i, n, n' and n'' (Schekman, Weiner and Kornberg, 1974, Meyer et al., 1978, Arai and Kornberg, 1979). The dnaB and dnaC proteins were originally purified by their ability to complement extracts prepared from thermosensitive mutants for øx174 replicative form synthesis and have native molecular weights of approximately

250,000 and 25,000 daltons, respectively (Wright, Wickner and Hurwitz, 1973, Wickner, Berkover, Wright and Hurwitz, 1973). The dnaB protein has a ribonucleoside triphosphatase activity which hydrolyzes all four ribonucleoside triphosphates to ribonucleoside diphosphates and inorganic phosphate and which is specifically stimulated by single-stranded DNA, but not by duplex DNA or RNA (Wickner, Wright and Hurwitz, 1974). The protein consists of a single polypeptide subunit of approximately 55,000 daltons suggesting that native dnaB is a tetramer (Ueda, McMacken and Kornberg, 1978). This is consistent with the observed intracistronic complementation between certain dnaB alleles in vivo (Lark and Wechsler, 1975). Although no enzymatic activities have yet been identified for the dnaC protein, the protein has been shown to interact physically with the dnaB protein, inhibiting the DNA-independent ATPase activity and forming a complex in the presence of ATP that is stable to gel filtration chromatography (Wickner and Hurwitz, 1975a).

It appears likely that replication factor Y and factor n' are the same protein since each displays an ATPase activity which is specifically stimulated by single-stranded øx174, but not fd or G4 (St-1), DNA (Wickner and Hurwitz, 1975b, Schlomai and Kornberg, 1980). The products of ATP hydrolysis are ADP and inorganic phosphate (Wickner and Hurwitz, 1975b). Recently, a unique DNA fragment of 55 nucleotides, isolated by exonuclease VII treatment of the largest Hae III fragment released from single-stranded øx174 DNA, was shown to be responsible for the specific stimulation of factor n' ATPase activity by øx174 DNA (Schlomai and Kornberg, 1980). As expected from its resistance to exonuclease VII digestion the fragment has the potential for forming a stable hairpin structure. This structure

is located in an intercistronic region on øx174 DNA between coat protein genes F and G, analagous to the position occupied by the origin of G4 replicative form synthesis (Sims and Dressler, 1978, Fiddes, Barrell and Godson, 1978).

The functions of the remaining factors is poorly understood at the present time. On the basis of kinetic evidence the initiation of øx174 replicative form synthesis has been shown to occur in at least two steps (Wickner and Hurwitz, 1974). The first stage requires the activity of the DNA binding protein, the dnaB and dnaC proteins, and replication factors X, Y and Z. These components form a complex with øx174 DNA in the presence of ATP that is stable to gel filtration chromatography (Wickner and Hurwitz, 1975c, Weiner, McMacken and Kornberg, 1976, Ray, Capon and Gefter, 1976). The next stage requires the dnaG protein and comprises the primer synthesizing step. When the prepriming complex has been prepared with purified proteins (DNA binding protein, dnaB, dnaC, i, n, n' and n") the dnaG protein may employ either ribonucleoside triphosphates or deoxyribonucleoside triphosphates plus ATP to catalyze the synthesis of a primer (McMacken, Ueda and Kornberg, 1977, McMacken and Kornberg, 1978). However, deoxyribonucleotides are required for effective priming by the dnaG protein if the prepriming complex has been prepared by incubating øx174 single-stranded with a crude extract and ATP and subsequently isolating the complex by gel filtration (Capon and Gefter, 1978). This difference may reflect the presence of additional factors in the crude extract which may degrade primers made exclusively with ribonucleotides, and thus raise the possibility that DNA chain synthesis in E. coli is also generally primed with deoxyribonucleotides rather than ribonucleo-

tides. This would explain the difficulty in observing the covalent attachment of RNA to the 5' end of nascent DNA chains in E. coli.

As reported for dnaG catalyzed oligonucleotide synthesis on G4 DNA:DNA binding protein complex, ATP but not dATP may be utilized to initiate the synthesis of oligonucleotides with the øx174 prepriming complex, and the presence of deoxyribonucleotide substrates inhibits ribonucleotide incorporation to a greater extent than expected from competition (McMacken and Kornberg, 1978). The øx174 DNA complex supports 5-10x as much oligonucleotide synthesis as does G4 DNA with DNA binding protein. In contrast to the unique site chosen for priming on G4 DNA, functional primers are synthesized from many distinct sites on øx174 DNA (McMacken, Ueda and Kornberg, 1977). This is based on the complexity of the fingerprint pattern obtained for a T_1 ribonuclease digest of the product synthesized in the presence of ribonucleotides only. This product ranges from 14 to 50 nucleotides in length, whereas the product synthesized with deoxyribonucleotides plus ATP is somewhat smaller.

The øx174 prepriming stage has been divided further into partial reactions which yield intermediates that may be isolated by agarose gel filtration (Wickner, 1978). In the first step, the DNA binding protein and replication factors Y and Z bind to øx174 DNA. In the next step the dnaB protein is transferred to this complex in a reaction that requires ATP, the dnaC protein and replication factor X. The dnaB protein binds to the complex, but the dnaC protein and replication factor X do not. It is this complex which presumably acts as the template for priming by the dnaG protein.

The prepriming stage has also been subdivided on the basis of studies with specific antibodies (McMacken, Ueda and Kornberg, 1977, Ueda, McMacken and Kornberg, 1978, McMacken and Kornberg, 1978). Addition of antibody directed against either the dnaB protein, DNA binding protein, factor i or factor n prevents the formation of the prepriming complex as measured by the ability to support subsequent primer synthesis and DNA synthesis. Once the complex has been formed, it is no longer sensitive to antibody directed against factors i or n, but remains sensitive to antibody directed against the other two proteins. These results have been interpreted to mean that factors i and n catalyze the formation of, but are not part of, the prepriming complex; however, the possibility remains that antibodies directed against these factors may not recognize their determinants when factors i and n are bound to the complex. In addition, since factor n has been resolved into three distinct activities (factors n, n' and n") subsequent to these studies (Meyer et al., 1978, Arai and Kornberg, 1979), it is not clear which activities are inactivated by the antibody directed against the combination.

These studies have also shown that the prepriming complex will immediately cease to support primer synthesis by the dnaG protein at any time after the addition of antibody directed against the dnaB protein, suggesting that the dnaB protein is continuously required. The length of the DNA product synthesized once priming has been allowed to proceed suggests that the prepriming complex promotes priming by the dnaG protein at regular intervals on the øx174 DNA template. On the basis of these results it has been proposed that the dnaB protein acts as a 'mobile promoter' which creates sites at which the dnaG protein may initiate primer

synthesis, by migrating along single-stranded øx174 DNA in the direction opposite that of DNA synthesis at the expense of energy derived from the hydrolysis of ATP (McMacken, Ueda and Kornberg, 1977).

The pathway described above is specific for øx174 DNA and does not occur with fd DNA (Wickner, 1978). One determinant of this specificity may be the site on øx174 DNA that stimulates the ATPase activity of replication factor Y (n'). Recently, a less specific priming pathway has been identified (Arai and Kornberg, 1980). In the absence of the DNA binding protein, the dnaG and dnaB proteins will initiate DNA synthesis on either øx174, M13 or G4 single-stranded DNA, or poly(dT), by synthesizing oligoribonucleotide primers, 10-60 residues in length. Furthermore, the dnaG and dnaB proteins will form a complex with øx174 DNA in the presence of ATP that is stable to gel filtration.

Two priming pathways for DNA synthesis on single-stranded DNA have been described that are catalyzed by phage-encoded priming proteins: the T_7 gene 4 protein, and the T_4 gene 41 and gene 61 proteins. In the presence of ribonucleoside triphosphates the multi-functional T_7 gene 4 protein enables the T_7 DNA polymerase to catalyze extensive DNA synthesis on either øx174 or fd single-stranded DNA (Scherzinger et al., 1977). E. coli DNA polymerase I may not replace the viral DNA polymerase suggesting that priming and DNA synthesis are specifically coupled. ATP and CTP together satisfy the requirement for ribonucleoside triphosphates in this reaction. When only ATP and CTP were present, it was demonstrated that a ribonucleotide primer, predominantly pppApCpCpA, was found at the 5' end of the DNA product synthesized on øx174 DNA. The same tetranucleotide is synthesized by the gene 4 protein alone on single-stranded DNA (Scherzinger, Lanka and

Hillenbrand, 1977).

In the absence of ribonucleoside triphosphates the gene 4 protein enables the T_7 DNA polymerase to synthesize a DNA product on nicked double-stranded T_7 DNA that is covalently attached to the DNA template (see below). Ribonucleoside triphosphates stimulate the synthesis of a DNA product that is not linked to the template, but rather is initiated de novo by the gene 4 protein (Hinkle and Richardson, 1975, Scherzinger et al., 1977, Richardson et al., 1978, Romano and Richardson, 1979a,b). The latter product, which may be labelled at its 5' end by including (γ-^{32}P) ATP, but not GTP, UTP or CTP in the reaction (Romano and Richardson, 1979a), has been shown to have oligoribonucleotide primers, primarily pppApCpCpC and pppApCpCpA, at its 5' end (Romano and Richardson, 1979b).

It is likely that oligoribonucleotide synthesis catalyzed by the gene 4 protein is template-directed since the random copolymer poly(dI,dT) will support primer synthesis, whereas homodeoxypolymers of dT, dA, dC, dI and dG, and poly(dC,dT) do not (Hillenbrand et al., 1978, Romano and Richardson, 1979a). There is no evidence to suggest that the gene 4 protein catalyzes the incorporation of deoxyribonucleotides as found for the dnaG protein.

The nucleotide sequences determined for the ribonucleotide primers synthesized by the gene 4 protein in vitro are consistent with those found for the RNA that is covalently attached to nascent T_7 DNA chains isolated in vivo (Okazaki et al., 1978, Seki and Okazaki, 1979). These nascent fragments were digested with the 3' to 5' exonuclease activity of T_4 DNA polymerase to yield mono- to pentaribonucleotides with a single deoxyribonucleotide residue at their 3' ends, and then labelled at the 5' end with

with (γ-^{32}P) ATP and T_4 polynucleotide kinase. The 5' portion of most tetra- and pentaribonucleotides obtained in this fashion is (^{32}p)ApC. In addition, analysis of tetra- and pentaribonucleotides with 5' triphosphate termini (labelled in vivo with H_3 (^{32}P)O_4), which presumably represent intact primers, reveals the general sequence pppApC(pN)$_{2-3}$ (Okazaki et al., 1978, Ogawa and Okazaki, 1979).

Initiation of DNA synthesis on single-stranded øx174, fd and T_4 DNA catalyzed by the T_4 DNA polymerase (gene 43 protein) and auxiliary proteins (T_4 genes 44/62 and gene 45 proteins) requires the T_4 gene 41 and gene 61 proteins and ribonucleoside triphosphates, and is stimulated by the T_4 DNA binding protein (gene 32 protein) (Liu et al., 1978, Silver and Nossal, 1978). In the absence of gene 32 protein the product synthesized with fd or øx174 DNA is full-length complementary strand DNA. Digestion of this product with a restriction endonuclease that cleaves either double-stranded fd or øx174 DNA at a single site, followed by denaturation, yields fragments of many lengths, suggesting that the gene 41 and gene 61 proteins initiate DNA synthesis at many sites along either template (Liu et al., 1978, Nossal, 1980). ATP and CTP will satisfy most of the ribonucleoside triphosphate requirement. The DNA product synthesized with fd DNA in the presence of (α-^{32}P) ATP was shown to have a pentaribonucleotide primer at its 5' end (Liu and Alberts, 1980).

In the presence of all four ribonucleoside triphosphates the gene 41 and gene 61 catalyze the synthesis of pentaribonucleotides with the common sequence pppApC(pN)$_3$ on both T_4 and øx174 single-stranded DNA in a rifampicin-resistant reaction (Liu and Alberts, 1980, Nossal, 1980). Thus, priming by the gene 41 and gene 61 proteins is quite similar to that observed

with the T_7 gene 4 protein. In the presence of ATP and CTP only, shorter oligoribonucleotides are synthesized as well suggesting that primer synthesis is template-directed and will terminate early if the template calls for incorporation of a residue which is missing from the reaction mixture.

As in the case of T_7, the sequence determined for the oligonucleotide primers synthesized in vitro corresponds to that found for the RNA which is covalently attached to the 5' end of nascent T_4 DNA chains isolated in vivo (Okazaki et al., 1978, Kurosawa and Okazaki, 1979). A substantial proportion of this RNA, analysed as described above for T_7 nascent chains, was found in pentaribonucleotides with the 5' terminal portion mostly (^{32}p)ApC.

In contrast to the initiation of DNA chain synthesis in E. coli which most likely occurs on a single-stranded region at the replication fork, the initiation of DNA replication at the chromosomal origin involves a double-stranded DNA substrate. As suggested by the requirement for transcription and the involvement of a greater number of dna gene products, the latter process is more complex. Whereas the initiation of DNA synthesis on single-stranded DNA appears to require only the formation of a primer, initiation on duplex DNA will generally also require the recognition of a specific origin, unwinding of the template strands (i.e., the establishment of a replication fork), and possibly the transfer of the replication machinery to the template strands, a process that may require additional factors. Two mechanisms for the initiation of DNA synthesis on double-stranded DNA have been identified. The first involves the nicking of one

of the template strands to expose a 3' OH terminus which may serve as a primer for DNA polymerase. Covalent extension of this primer will result in the displacement of that portion of the same template strand which bears the 5' phosphorylated terminus. This mechanism has been found for the initiation of leading strand synthesis on duplex T_7, T_4 and øx174 RF I DNA. Initiation of DNA synthesis on the displaced strand, which becomes the lagging strand, requires the de novo synthesis of a primer by one of the pathways described above. The second mechanism, observed for the ColE1 plasmid, involves transcription by RNA polymerase at a specific site, thus accomplishing in one stroke the recognition of a specific origin, unwinding of the duplex, and priming. This process is considered to be a simpler version of what may possibly happen at the E. coli chromosomal origin. A brief discussion of these pathways follows.

The overall replication of the single-stranded DNA phages of E. coli occurs in three distinct stages: 1) conversion of the (+) strand DNA (the single-stranded viral DNA) to the closed-circular replicative form, RF I (the initiation of this stage was described earlier), 2) synthesis of progeny RF I molecules, and 3) synthesis of circular (+) strand DNA from RF I. A reaction resembling the final stage for øx174 RF I DNA has been reconstituted in vitro with purified proteins: the øx174 gene A protein, the E. coli rep protein, DNA binding protein, DNA polymerase III and elongation factors (Eisenberg, Scott and Kornberg, 1976a,b, Sumida-Yasumoto, Yudelevich and Hurwitz, 1976). The role of the gene A protein is to nick the (+) strand of a duplex øx174 RF I molecule at a specific site, thus creating a 3' OH terminus at which DNA synthesis is initiated (Ikeda, Yudelevich and Hurwitz, 1976, Henry and Knippers, 1974). The gene A protein

becomes covalently attached to the 5' phosphorylated terminus created at the nick (Eisenberg, Scott and Kornberg, 1978, Sumida-Yasumoto et al., 1978). DNA synthesis proceeds by covalent extension of the 3' OH terminus as the 5' phosphoryl strand, which bears the gene A protein, is displaced from the template by ATP-dependent rep protein catalyzed unwinding (Scott et al., 1977, Duguet, Yarranton and Gefter, 1978). The overall mechanism is similar to that proposed by the rolling-circle model (Gilbert and Dressler, 1968). The end of the displaced strand does not become free, but rather, remains 'attached' to the template at the site of unwinding (Eisenberg, Griffith and Kornberg, 1977). This is based on the analysis of replicative intermediates by electron microscopy, which appear as a single-stranded DNA circle, complexed to DNA binding protein, that is attached at a single site to a full-length duplex circle.

This attachment is probably due to a non-covalent interaction between the gene A protein molecule covalently linked to the 5' end of the displaced strand and the rep protein molecule which is bound to the template at the site of unwinding (Duguet, Yarranton and Gefter, 1978, Yarranton and Gefter, 1979). This was deduced from the observation that the rep protein cannot unwind a DNA strand from a nick unless that nick was introduced by the gene A protein, which presumably must remain bound to the 5' phosphoryl terminus. Once DNA synthesis has proceeded around the template, a circular (+) strand product is generated from the displaced strand by a transfer reaction catalyzed by the gene A protein (Eisenberg, Scott and Kornberg, 1978, Sumida-Yasumoto et al., 1978).

Recently, the synthesis of progeny ϕx174 RF (stage two, RF → RF) has been accomplished in vitro by coupling two reactions: the synthesis of

(+) strand DNA from RF I as just described, followed by the conversion of the displaced (+) strand to duplex replicative form by the DNA binding protein, the dnaG, dnaB and dnaC proteins, factors i, n, n' and n", and DNA polymerase III and elongation factors as described earlier (Arai et al., 1979). In contrast, another group has shown that the synthesis of (+) strand DNA from øx174 RF I in crude extracts requires the dnaG, dnaB and dnaC proteins in addition to the rep and gene A proteins (Sumida-Yasumoto and Hurwitz, 1977, Sumida-Yasumoto et al., 1978). In vivo evidence suggests that the dnaG, but not dnaB and dnaC, gene products are required in this process (see Dumas, 1978).

The coupled system for progeny øx174 RF synthesis described above also predicts that synthesis of the leading strand, or (+) strand, will be continuous, whereas synthesis of the lagging strand will be discontinuous (Arai et al., 1979). This contradicts in vivo studies that have shown that synthesis of progeny RF involves discontinuous synthesis on both strands (Machida, Okazaki and Okazaki, 1977, Baas, Teerstra and Janz, 1978).

As mentioned earlier, in the absence of ribonucleoside triphosphates the T7 gene 4 protein enables the T7 DNA polymerase to catalyze extensive DNA synthesis on a duplex template (Hinkle and Richardson, 1975, Scherzinger and Klotz, 1975). This reaction is specific for the homologous polymerase and does not occur with E. coli DNA polymerases I, II or III, or the T4 DNA polymerase. All of the DNA product synthesized in the absence of ribonucleoside triphosphates is covalently attached to the template, suggesting that synthesis is initiated at the 3' OH terminus created by a nick in the template (Kolodner and Richardson, 1978). This was confirmed by the demonstration that duplex DNA repaired with DNA polymerase and polynucleotide

ligase will no longer support DNA synthesis by the T7 DNA polymerase with the gene 4 protein unless nicks are introduced into the template by nuclease (Kolodner et al., 1978). It was also shown that the gene 4 protein does not stimulate DNA synthesis by the T7 DNA polymerase on single-stranded DNA.

These results are consistent with the idea that the gene 4 protein catalyzes DNA unwinding, which has been substantiated by studies on the nucleoside triphosphatase activity of the protein (Kolodner and Richardson, 1977, Hillenbrand et al., 1978). In a reaction that requires single-stranded DNA the gene 4 protein hydrolyzes both ribo- and deoxyribonucleoside triphosphates to nucleoside diphosphates and inorganic phosphate. As in the case of DNA synthesis catalyzed by the T7 DNA polymerase, this reaction will also occur with duplex DNA but only in the presence of the other protein. Under these conditions DNA synthesis is accompanied by NTP hydrolysis. Both reactions are coupled in that the addition of ddTTP, a DNA synthesis inhibitor, also reduces the rate of hydrolysis by the gene 4 protein, while the addition of an inhibitor of the hydrolysis reaction, β,γ-methylene-dTTP, inhibits DNA synthesis as well.

Together, these results suggest that the gene 4 protein and T7 DNA polymerase catalyze leading strand synthesis, initiated from a 3' OH group at a nick, in a continuous fashion. As discussed earlier, recent studies have suggested that synthesis on the displaced strand, or lagging strand. is initiated by a ribonucleotide primer synthesized de novo by the gene 4 protein (Richardson et al., 1978, Romano and Richardson, 1979).

Semiconservative replication of superhelical ColE1 DNA is catalyzed by a partially purified cell extract in a reaction that requires all four

ribonucleoside triphosphates and is inhibited by rifampicin (Sakakibara and Tomizawa, 1974a). The products of this reaction are fully replicated molecules of the natural superhelical density. In the presence of glycerol an early replicative intermediate accumulates: the 6S L-strand fragment (complementary to the H-strand) (Sakakibara and Tomizawa, 1974b). This intermediate may be chased into fully replicated product by lowering the glycerol concentration in the reaction. Synthesis of 6S L-strand DNA is inhibited by rifampicin, suggesting that it is primed by RNA polymerase, but subsequent DNA synthesis, which presumably includes the initiation of H-strand synthesis, is not (Tomizawa, 1975). The point of transition from RNA primer to DNA product has been established by analyzing purified 6S L-strand DNA fragments that were treated with alkali to remove the RNA (Tomizawa, Ohmori and Bird, 1977). Transition occurs at any one of three sites also found for nascent 6S L-strand DNA isolated *in vivo* (Bolivar et al., 1977).

Most of the 6S L-strand fragments isolated *in vitro* have only a single AMP residue at their 5' ends (Bird and Tomizawa, 1978). Thus, it has not been possible to learn where RNA polymerase begins synthesis of the primer, or what its length or structure might be. Most likely, RNase H is responsible for the efficient removal of the primer once DNA synthesis is initiated. This is inferred from the requirement for RNase H in order to observe specific initiation of ColE1 DNA synthesis in a purified system consisting of RNA polymerase and DNA polymerase I (Itoh and Tomizawa, 1978).

DNA synthesis subsequent to that of 6S L-strand fragments has been reported to involve the products of the *dnaB*, *dnaC* and possibly *dnaG* genes (see Tomizawa and Selzer, 1979). Thus, it is possible that initiation

of H-strand (or lagging strand) synthesis proceeds via the same pathway identified for øx174 single-stranded DNA.

Focus of this thesis

The experiments reported in the first part of this thesis were concerned with determining conclusively that the ability to prime DNA synthesis on øx174 and G4 single-stranded phage DNA resides with the dnaG protein as suggested by earlier reports for G4 DNA (Bouche, Zechel and Kornberg, 1975). For this purpose, a thermolabile dnaG protein prepared from a dnaG^{ts} strain of E. coli was utilized. This approach made it possible to separate the priming event from DNA synthesis and thus demonstrate that dNTPs as well as rNTPs serve as substrates for priming (Capon and Gefter, 1978). Similar findings were reported by two other groups that demonstrated that the dnaG protein catalyzes the synthesis of oligonucleotide primers with rNTPs and dNTPs (Wickner, 1977, McMacken et al., 1977).

The second part of this thesis represents an effort to characterize the primer synthesizing reaction. Of particular concern has been the sequence and structure of the primers and the template that directs their synthesis, the mechanism of primer initiation and completion, the contribution of ribo- and deoxyribonucleotides to primer formation, and the nature of the transition between the priming event and DNA synthesis.

Materials

Bacterial strains: E. coli H562 (F^+ thy$^-$ polA1$^-$ endo I$^-$) was used in the preparation of cell-free extracts for in vitro øx174 replicative form synthesis, and for the purification of the dnaG protein, DNA polymerase III*, copolymerase III*. E. coli NY73 (leu$^-$ met$^-$ thy$^-$ str^r rif^r polA1$^-$ dnaG3^{ts}) was used in the preparation of the receptor extract for the dnaG complementation assay, and for the purification of the dnaG^{ts} protein. E. coli K12 (Grain Processing Co., Muscatine, Iowa) was used for the purification of DNA polymerase I and DNA binding protein. E. coli C (HF 4704 thy$^-$ su$^-$ uvrA$^-$) and E. coli K12 (MM 28, W3350 gal$^-$ str^r) were used for the growth and preparation of øx174, G4 and α3, and St-1 and øK single-stranded phage DNA, respectively. S. typhymurium D7000 (su$^-$) was used for the growth and preparation of (^{3}H) P22 double-stranded phage DNA.

Phage strains: øx174 was the am3 strain. G4 (Godson, 1974) was obtained from Dr. G. Godson. St-1 (Bradley, 1970) was obtained from Dr. S. Wickner. øK (Taketo, 1976) and α3 (Bradley, 1962) were obtained from Dr. J. Sims. P22 13$^-$ amH101 was obtained from Dr. F. Winston.

Enzymes and proteins: Lysozyme (egg albumin)- Sigma Chemical; bovine serum albumin- Schwartz-Mann; ribonucleases T_1, T_2 and U_2- Calbiochem; pancreatic DNase and RNase A, snake venom phosphodiesterase, calf spleen phosphodiesterase- Worthington; T4 polynucleotide kinase, E. coli alkaline phosphatase- Boehringer-Mannheim; restriction endonuclease Hae III- New England Biolabs; Physarum I and B. cereus ribonuclease were kindly provided by Dr. H. Donis-Keller

Nucleotides: dATP, dGTP, dCTP and dTTP- Schwartz-Mann Biochemicals; ddTTP- PL Biochemicals; (α-^{32}P) dATP, dGTP, dCTP and dTTP (300-400 Ci/ mmmole), and (^{3}H) dTTP (50-100 Ci/ mmole)- New England Nuclear; ATP, GTP, CTP, UTP, 3' dAMP, 3' dAMP, 3' dGMP, ApG and 5' adenosine tetraphosphate- Sigma Chemical; (α-^{32}P) ATP, GTP, CTP and UTP (200-300 Ci/mmole), and (γ-^{32}P) ATP (2000-3000 Ci/mmole)- ICN Pharmaceuticals; ApGpG was kindly provided by Dr. H. G. Khorana.

Polynucleotides: E. coli B tRNA- Schwartz-Mann Biochemicals; calf thymus and salmon sperm DNA- Worthington; poly(dT)- Miles Laboratories; (^{3}H) poly(dA) (150 cpm/pmole) and phage fd single-stranded DNA were kindly provided by Dr. I. Molineux and Dr. L. Sherman, respectively; St-1 RF I DNA was kindly provided by Dr. J. Sims; (^{3}H) P22 DNA (20 cpm/pmole) was prepared as previously described (Ray et al., 1974).

Methods

Preparation of DNA

øx174 single-stranded viral DNA: øx174 am3 phage was grown on *E. coli* C (HF 4704 *su*$^-$) and purified as previously described (Sinsheimer, 1966), except that a discontinuous CsCl density gradient followed by a continuous CsCl gradient were substituted for the phase partition step. After dialysis in 50 mM sodium tetraborate the phage preparation was made 0.5% in Sarkosyl, warmed to 70°C, gently extracted with warm phenol 3 times, once with ether, and the aqueous phase was then dialysed extensively against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA.

G4, St-1, øK and α3 single-stranded phage DNA: Host cells (*E. coli* K12 for St-1 and øK, *E. coli* C for G4 and α3) were grown at 37°C in TK media (per liter, 10 g bactotryptone, 5 g KCl, 10 mM $MgSO_4$ and 20 µg/ml thymine) to a density of 0.7-0.8 x 10^9 cells/ml. $CaCl_2$ was added to 2 mM, and cells were infected at a m.o.i. of 0.05. Once the cell density began to fall (30-45 minutes post-infection) the cells were rapidly chilled to 5°C in a dry ice/ethanol bath and collected by centrifugation. The pellet, which consisted of whole cells and some debris, usually contained half of the recoverable phage titer (2-5 x 10^{10} pfu/ml culture), and was processed as follows. The pellet was resuspended in 1/200 volume of 0.25 M Tris-HCL (pH 8.0), 10 mM EDTA and incubated with 1 mg/ml lysozyme on ice. After 45 minutes the mixture was warmed to RT, sarkosyl was added to 0.1% and the mixture was then vortexed thoroughly. After 15 minutes CsCl was added to

a density of 1.4 g/ml and the mixture was purified by two successive CsCl density gradients. After the phage band was collected and dialysed in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, the phage were pelleted (1 hr at 49,000 rpm in the Beckman SW 50.1 rotor) and resuspended in the same buffer to a concentration of 1-5 x 10^{14} / ml, assuming that 1 OD_{260} = 1.3 x 10^{13} particles/ml as for øx174 (Sinsheimer, 1966). The phage were extracted gently with phenol three times, twice with ether, and the ether then removed by bubbling N_2 through the solution. The phage DNA, which was generally > 80% circular as determined by electron microscopy (kindly performed by Dr. Don Ross), was further purified by velocity sedimentation on a 5-20% alkaline sucrose gradient (0.25 M NaOH, 1 M NaCl, 1 mM EDTA). Full-length circular DNA sedimented halfway through a gradient centrifuged at 49,000 rpm for 5 hr in the Beckman SW 50.1 rotor, and was detected by absorbtion at 260 nm.

Preparation of enzymes and proteins

dnaG protein was prepared by a modification of a published procedure (Wickner, Wright and Hurwitz, 1973) communicated by Dr. Sue Wickner. All steps were performed at 0°C. 500 g of E. coli H562, grown and frozen in an equal volume of 50 mM Tris-HCl (pH 7.5) containing 10% sucrose as previously described (R. Wickner et al., 1972), were thawed, made up to 0.15 M NaCl, 10 mM EDTA and 2 mM dithiothreitol, and lysed by the addition of 1 mg/ml lysozyme. After 30 minutes at 0°C the mixture was centrifuged at 100,000 for 30 minutes. The supernatant was adjusted to pH 6.5 with 1 M Tris base, and nucleic acid was then precipitated by the addition of

a freshly prepared 20% streptomycin sulfate solution to 4%. After 3 minutes the mixture was centrifuged at 20,000 x g for 15 minutes, and the supernatant was adjusted to 45% saturation with solid ammonium sulfate (25.8 g/100 ml). After 5 minutes the protein precipitate was collected by centrifugation at 20,000 x g for 20 minutes, and washed successively with 500 ml of a 40% and 20% saturated ammonium sulfate solution in buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol). The supernatant obtained after the 20% wash was brought to 50% saturation with solid ammonium sulfate, and the protein precipitate was collected by centrifugation and dissolved in 50 ml of buffer A (Fraction 1, 2500 units, 740 mg protein). Fraction 1 was dialysed for 2 hr against 2 changes of buffer A, diluted to 300 ml with buffer A (< 0.02 M ammonium sulfate), and applied to a column of DEAE-cellulose (DE 52, 3.7 x 35 cm). The column was washed with 200 ml of buffer A, and dnaG activity was then eluted with a linear gradient of buffer A containing 0-0.35 M KCl. Active fractions (> 30 units/mg protein) eluting at 0.12 M KCl, were combined, and the protein precipitated with 60% ammonium sulfate, centrifuged and redissolved in 3.0 ml of buffer B (buffer A containing 50 mM Tris-HCl, pH 7.5) (Fraction 2, 2060 units, 45 mg protein). Fraction 2 was dialyzed for 2 hr against two changes of buffer B, diluted with two volumes of buffer B (< 0.06 M ammonium sulfate) and applied to a column of DNA-agarose (2.8 x 10 cm). The column was washed with two volumes of buffer B, and dnaG activity was then eluted with buffer B containing 1 M NaCl. Active fractions were combined, and the protein was precipitated with 60% ammonium sulfate, centrifuged and redissolved in 1 ml of buffer A (Fraction 3, 1250 units, 9 mg protein). Fraction 3 was dialysed for 2 hr against two changes of buffer A (= 0.05 M

NaCl equivelant) and applied to a column of phosphocellulose (1.7 x 7 cm). dnaG activity passed through the column as it was washed with buffer A. Active fractions were precipitated with 75% ammonium sulfate, centrifuged and redissolved in 0.5 ml buffer A (Fraction 4, 300 units, 0.3 mg protein). Fraction 4 was dialysed for 2 hr against two changes of buffer A containing 1 M KCl and applied to two 14 ml 15-35% glycerol gradients (Beckman SW 40 Ti rotor) in the same buffer. After 67 hour at 40,000 rpm active fractions (0.4 ml each) were combined, dialysed against buffer A and concentrated by DEAE-cellulose chromatography to a final volume of 0.15 ml in buffer A containing 0.5 M KCl (Fraction 5, 260 units, <0.07 mg protein). Fraction 5, which had a specific activity of 3000-5000 units/mg, was used for the experiments described in Part two of this thesis.

E. coli DNA binding protein was purified to apparent homogeneity according to a published procedure (Molineux, Friedman and Gefter, 1974) with the help of Drs. G Yarranton, M. Duguet and R. Das. Exonuclease I and RNAse H activity were completely separated from the DNA binding protein by DEAE-Sephadex chromatography as described. 2.5 kg of E. coli K12 yielded 30 mg of pure protein. The protein was concentrated by precipitation with 70% saturated ammonium sulfate solution, dissolved in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM β-mercaptoethanol and 10% glycerol, at a concentration of 0.4-0.7 mg/ml to prevent aggregation, and dialysed in the same buffer. Exonuclease I and RNAse H assays, which measured the release of acid-soluble nucleotide from (^{3}H) P22 single-stranded DNA and (^{3}H) polyA:poly(dT), respectively, were performed as previously described (Molineux, Friedman, and Gefter, 1974). The elution behavior of the DNA binding protein was

followed by electrophoresis on a 12.5% polyacrylamide gel containing 0.1% sodium dodecyl sulphate as previously described (Maizel, 1971).

E. coli DNA polymerase I was purified to apparent homogeneity by modifications of existing procedures with the help of Drs. G. Yarranton and R. Das. 500 g. of E. coli K12 was lysed in a French press, centrifuged at 150,000 x g for 90 minutes, and the resulting supernatant passed through columns of phosphocellulose and DEAE-cellulose (to remove nucleic acid) as previously described (Kornberg, and Gefter, 1971). This fraction was successively purified by ammonium sulfate fractionation and DEAE-cellulose and phosphocellulose chromatography (Jovin, Englund and Bertsch, 1969) and chromatography on hydroxyapatite (Richardson, 1966). The final preparation had a specific activity of greater than 20,000 units/mg when assayed on activated calf thymus DNA (see below), and was stored in 0.1 M potassium phosphate (pH 7.0), 1 mM β-mercaptoethanol, 1 mM EDTA and 50% glycerol at -20^{o}C.

DNA polymerase III*, copolymerase III* and dnaG protein (used for the experiments described in Part one) were purified by a modification of existing procedures that yielded all three activities from the same cells, E. coli H562, as previously described (Capon and Gefter, 1978). Thermolabile $dnaG^{ts}$ protein was also purified by this procedure, starting with E. coli NY73, which is summarized as follows. Cells were gently lysed, the resulting high speed supernatant was passed through a DEAE-cellulose column to remove nucleic acid, precipitated with 45% ammonium sulfate solution, and the precipitate was washed successively with 40%, 30% and

20% ammonium sulfate solution as previously described (Ray, Capon and Gefter, 1976). The supernatant resulting from the 20% wash contained at least 50% of each protein activity and was brought to 50% saturation with ammonium sulfate. The precipitate was dissolved in buffer A (50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 1 mM EDTA and 20% glycerol), dialysed and applied to a phosphocellulose column. Copolymerase III* activity flowed through the column and was further purified by DEAE-cellulose and Sephadex G-150 chromatography as previously described (W. Wickner, 1973). The dnaG protein and DNA polymerase III* eluted from the phosphocellulose column at 0.075-0.15 M NaCl together, and were resolved by chromatography on DEAE-Sephadex, with dnaG activity eluting at 0.11 M NaCl and pol III* at 0.15 M NaCl. Each protein was purified further by phosphocellulose chromatography at pH 8.0. All three activities were judged to be free from one another by the requirement shown for each in a reaction which catalyzed replicative form synthesis with G4 single-stranded DNA and DNA binding protein (see Table 1B).

E. coli RNA polymerase used for the assay of DNA polymerase III* and copolymerase III* was purified to apparent homogeneity according to a published procedure (Burgess and Jendrisak, 1975) with the help of Drs. G. Yarranton and R. Das. 500 g of E. coli K12 yielded greater than 30 mg of pure protein with a specific activity of greater than 1700 units/mg when assayed on calf thymus DNA substrate as described (Burgess, 1969).

Preparation of extracts

Receptor extract for dnaG assays was prepared by a modification of a published procedure (Wickner, Wright and Hurwitz, 1973) communicated by Dr. Sue Wickner. E. coli NY73 was grown to an OD_{595} of 0.5-1.0 at 30°C in Hershey broth (per liter, 10 g Nutrient broth, 5 g Bactopeptone, 5 g NaCl, 1 g glucose) plus 20 µg/ml thymine. Cells were collected by centrifugation at room temperature, resuspended in an equal volume of 50 mM Tris-HCl (pH 7.5) and 10% sucrose and quick frozen in liquid N_2. Cells were thawed and lysed, and a high speed supernatant was prepared, treated with streptomycin sulfate, and precipitated with 45% ammonium sulfate as described for the dnaG protein purification procedure. The protein precipitate was dissolved in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol and 10% glycerol in a volume sufficient to give a final protein concentration of 20-30 mg/ml, dialysed for 2 hr against the same buffer, and quick frozen in liquid N_2.

Cell-free extracts for preparation of 'activated' øx174 DNA were prepared as previously described (Ray, Capon and Gefter, 1976). Extracts were prepared from E. coli H562 (dna^+) as described above for the dnaG assay receptor except that nucleic acid was removed from the extract by passing it over a DEAE-cellulose column in 0.4 M ammonium sulfate instead of treatment with streptomycin sulfate. The final preparation was dissolved in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM dithiothreitol and 10% sucrose.

Enzyme assays

dnaG complementation assay (25 µl) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, 25 µg/ml rifampicin, 2 mM ATP, 50 µM each dATP, dGTP, dCTP and (^{3}H) dTTP (1000 cpm/pmole), 400 pmole (as nucleotide) øx174 single-stranded viral DNA, 5 µl receptor extract (20-30 mg/ml of protein) prepared from NY73 (dnaG^{ts}) cells that was inactivated by heating at 38°C for 10-20 minutes, and the fraction to be assayed. After 20 minutes at 30°C the reaction was stopped with 1 ml 5% trichloroacetic acid, and the acid-insoluble material was collected on Whatman GF/C filters. Incorporation was determined by liquid scintillation counting. One unit of dnaG protein catalyzes the incorporation of 1 pmole dTMP in 20 minutes under these conditions (Wickner, Wright and Hurwitz, 1973).

DNA polymerase assay (0.15 ml) contained 67 mM Tris-HCl (pH 7.4), 6.7 mM $MgCl_2$, 0.2 M KCl, 3.3 mM β-mercaptoethanol, 33 µM each dATP, dGTP, dCTP and (^{3}H) dTTP (250 cpm/ pmole), 15 nmoles of 'activated' calf thymus DNA substrate, and fraction to be assayed. After 5 minutes at 30°C the reaction was stopped and incorporation determined as described above (see dnaG assay). One unit of DNA polymerase catalyzed the incorporation of 1 nmole dTMP in 5 minutes under these conditions (Kornberg and Gefter, 1972).

DNA polymerase III* and copolymerase III* were assayed in a reaction which required both activities to observe DNA synthesis (W. Wickner et al., 1973, Hurwitz, Wickner and Wright, 1973). Assays (25 µl) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 4 mM dithiothreitol, 0.5 mg/ml bovine serum albumin,

5 mM ATP, 0.1 mM GTP, CTP and UTP, 0.04 mM dATP, dGTP, dCTP and (^{3}H) dTTP (1000 cpm/pmole), 1.2 mM spermidine, 0.5 nmoles (as nucleotide) fd single-stranded DNA, 1 µg DNA binding protein, fractions containing DNA polymerase III* and copolymerase III* activity, and was initiated by the addition of 0.3 units RNA polymerase. After 20 minutes at 30°C the reaction was terminated by the addition of 0.05 ml of 1 mg/ml salmon sperm DNA and 1 ml of 5% trichloroacetic acid. dTMP incorporation was determined as described for the dnaG assay. One unit of either DNA polymerase IIII* or copolymerase III* stimulated the incorporation of 1 nmole dTMP in the presence of saturating amounts of the other protein (W. Wickner, 1973).

Assays for in vitro DNA synthesis and primer synthesis

Synthesis of G4, St-1, ØK and α3 replicative form (RF II) (in Part two) Reaction mixtures (25 µl) contained 20 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 4 mM dithiothreitol, 20 µg/ml rifampicin, 0.5 mg/ml bovine serum albumin, 0.2 mM ATP, 0.04 mM dATP, dGTP, dCTP and (^{3}H) dTTP (1000 cpm/pmole), 0.4 nmoles (as nucleotide) single-stranded phage DNA, 0.04 units dnaG protein, 0.35 µg DNA binding protein and 1.5 units DNA polymerase I. After 20 minutes at 30°C reactions were terminated and dTMP incorporation determined as described for dnaG assays.

dnaG catalyzed oligonucleotide synthesis was performed as previously described (Wickner, 1977). Reaction mixtures (50 µl) contained 50 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 1 mM dithiothreitol, 20 µg/ml rifampicin,

1 mg/ml bovine serum albumin, the indicated concentration of ribo- and deoxyribonucleoside triphosphates, 4 nmoles (as nucleotide) single-stranded phage DNA, 3 µg DNA binding protein and 1 unit dnaG protein. After 20 minutes at 30°C each reaction mixture was filtered through a Sepharose 6B column (0.6 x 20 cm) at room temperature in 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin and 5% glycerol. The product eluted in the void volume with the template DNA away from unreacted sustrates and the yield of incorporated (α-^{32}P) or (γ-^{32}P) nucleoside triphosphate was determined by measuring the cerenkov radiation. The product was further purified by extraction with phenol twice, extraction with ether once, and precipitated by the addition of 1 µg tRNA carrier, 0.1 volume sodium acetate, pH 5.2, and 2.5 volumes of ethanol. After 15 minutes at -70°C the precipitate was collected by centrifugation at 10,000 x g for 15-30 minutes, lyophilized to dryness, and dissolved in 5-10 µl of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. This material was analyzed by electrophoresis on polyacrylamide gels or nucleic acid sequencing as described below.

Synthesis of øx174 and G4 replicative form (RF II) (in Part one)
Reaction mixtures (50 µl) contained 20 mM Tris-HCl (pH 7.5); 12 mM $MgCl_2$; 4 mM dithiothreitol; 2 mM KH_2PO_4; 30 µg/ml rifampicin; 0.5 mg/ml bovine serum albumin; either 0.1 nmole activated øx174 DNA (see below) or 0.05 nmole G4 DNA plus 0.1 µg DNA binding protein and 0.2 mM ATP; 0.05 units dnaG protein or dnaG^{ts} protein; 0.1 units DNA polymerase III*; 0.1 units copolymerase III*; and 0.04 mM each dATP, dGTP, dCTP and (^{3}H) dTTP (1000 cpm/pmole). After 20 minutes at 30°C reactions were terminated and dTMP

incorporation was determined as described for DNA polymerase III* assays. Two stage reactions were performed as described above except that the indicated deoxyribonucleoside triphosphates were omitted from the first incubation and added during the second incubation.

Preparation of 'activated' øx174 DNA (for the reaction described above) was as previously described (Ray, Capon and Gefter, 1976). Reaction mixtures (1 ml) contained 20 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 4 mM dithiothreitol, 30 µg/ml rifampicin, 2 mM ATP, 2 nmoles (as nucleotide) øx174 single-stranded DNA, and 0.1 ml cell-free extract prepared from E. coli H562 (20-30 mg/ml protein). After 10 minutes at 30°C the mixture was filtered through a Biogel column (0.6 x 25 cm) at 4°C in 20 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 4 mM dithiothreitol, 0.5 mM ATP and 2.5% sucrose. Activated øx174 DNA, which eluted ahead of the bulk protein, was assayed as follows. An aliquot (50 µl) from each column fraction (0.5 ml) was incubated with 0.04 mM each dATP, aGTP, dCTP and (^{3}H) dTTP (10,000 cpm/pmole), and 1 µl of cell-free extract. This amount of extract was sufficient to catalyze replicative form synthesis with activated øx174 DNA, but not unreacted øx174 DNA (Ray, Capon and Gefter). After 20 minutes at 30°C the reaction was terminated and dTMP incorporation was measured. Active fractions containing the activated øx174 DNA were used in the experiments described above.

Sequencing and electrophoresis procedures

Denaturing polyacrylamide gel electrophoresis was performed as described (Maxam and Gilbert, 1977). Slab gels (0.4-1.5 mm thick) consisted of 4%, 10% or 20% (wt/vol) acrylamide-bisacrylamide (30:1), 7 M urea, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, and were polymerized with 3 mM ammonium persulfate and 0.06% TEMED. Samples were denatured by heating at 100°C for 1.5 minutes in 5 M urea and 0.05% each bromophenol blue and xylene cyanol, applied to the gel, and electrophoresed at 20-40 volts/cm at room temperature. Products were visualized by autoradiography with Kodak XR-5 film, using Dupont Cronex Lightning-plus intensifying screens (at -70°C) when necessary. Products were eluted from the gel as described (Maxam and Gilbert, 1977) with 0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1 mM EDTA and 0.1% sodium dodecyl sulfate, for 1-12 hrs at 37°C, precipitated by the addition of three volumes ethanol, reprecipitated from 0.3 M sodium acetate with three volumes ethanol, rinsed with ethanol and dried by lyophilization. Ethanol precipitation was carried out with 1 µg/ml carrier tRNA at -70°C for 15 minutes, and the precipitate collected by centrifugation at 10,000 x g for 15-30 minutes.

RNA sequencing: Digestion with T_1 ribonuclease was performed in a reaction mixture (5 µl) that contained 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 25 units enzyme and 25 µg carrier tRNA for 1.5 hr at 37°C. Alkali hydrolysis was carried out in a reaction mixture (10 µl) that contained 0.2 M NaOH and 25 µg carrier tRNA for 16 hrs at 37°C. The two-dimensional electrophoresis procedure, elution of nucleotides from the fingerprint, and subsequent

compositional analysis are described by Barrell (1971). Electrophoresis on DEAE-81 paper was performed at pH 3.5 (pyridinium-acetate buffer) or pH 1.9 (2.5% formic acid, 8.7% acetic acid, v/v). Digestion with E. coli alkaline phosphatase was performed in a reaction mixture (10 µl) that contained 50 mM Tris-HCl (pH 8.0) and 1 µg enzyme. After 60 minutes at 55°C the enzyme was removed by three phenol extractions, and the product was recovered by ethanol precipitation from 0.3 M sodium acetate (see above). Partial digestion with snake venom phosphodiesterase was performed in a reaction mixture (10 µl) that contained 10 mM Tris-HCl (pH 8.0) and 5-20 mg enzyme. After 2, 5, 10, 20 and 40 minutes at 37°C, a 2 µl aliquot was removed and the enzyme inactivated by adding an equal volume of 2 mM EDTA and heating at 100°C for 2 minutes. Digestion of the product was followed by homochromatography. Two-dimensional analysis of partial venom digests by electrophoresis on cellulose-acetate at pH 3.5 and homochromatography was performed as described (Barrell, 1971, Brownlee and Sanger, 1969, Sanger et al., 1973). The homomixture was prepared as described (Silberklang et al., 1977). RNA sequencing by partial digestion with base-specific ribonucleases was performed as described (Donis-Keller et al., 1977, Lockard et al., 1978). Partial enzymatic digests were performed in reaction mixtures (20 µl) that contained 20 mM sodium citrate (pH 5.0), 1 mM EDTA, 7 M urea, 0.25 µl/ml carrier tRNA, 0.025% of bromophenol blue and xylene cyanol, and either 0.01-0.1 units T_1, 0.05-1.0 units U_2, or 1-3 µl of a Physarym I supernatant fraction, for 15 minutes at 50°C. Partial digestion with B. cereus ribonuclease was performed in a reaction mixture (10 µl) that contained 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 µg carrier tRNA and 0.02-0.1 units enzyme. After 15 minutes at 50°C, 10 µl

of 10 M urea and 0.05% each bromophenol blue and xylene cyanol were added. Partial alkali hydrolysis was performed in a reaction mixture (10 µl) that contained 50 mM sodium carbonate (pH 9.0), 1 mM EDTA and 0.25 µg/ml of carrier tRNA. After 15-60 minutes at 90°C an equal volume of the urea-dye mixture was added. Each partial digestion was heated at 90°C for 2 minutes ana applied to a 20% polyacrylamide gel containing 7 M urea (see above). Two-dimensional polyacrylamide gel electrophoresis was performed as described (DeWachter and Fiers, 1972, Lockard et al., 1978). The slab gel for the first dimension consisted of 10% acrylamide-bisacrylamide (20:1), 7 M urea and 25 mM citric acid (pH 3.5) and was polymerized by the addition of (per 150 ml) 0.5 ml of 0.25% $FeSO_4:7H_2O$, 0.5 ml of 10% ascorbic acid and 0.05 ml of 30% H_2O_2. After electrophoresis at 10 volts/cm for 24 hr, the gel strip containing the sample was cut out and polymerized into the second dimension gel which consisted of 20% polyacrylamide (pH 8.3) containing 7 M urea as described above. The procedure used to release either 3' AMP or 3' dAMP from the T_1 nucleotide of composition ppp(A or dA)pGp was as follows. The nucleotide was dephosphorylated in a reaction mixture (3 µl) that contained 50 mM Tris-HCl (pH 7.5), 0.15 OD_{260} units carrier ApG and 0.005 units E. coli alkaline phosphatase. After 60 minutes at 65°C the enzyme was inactivated by the addition of 0.1 M nitriloacetic acid. After 15 minutes at room temperature the mixture was boiled for 2 minutes. The nucleotide was then digested with calf spleen phosphodiesterase in a reaction mixture (8 µl final volume) that contained 5 mM potassium phosphate, 0.1 M ammonium acetate, 0.02 M acetic acid (final pH 5.5-6.0) and units of enzyme. After 2 hr at 37°C an aliquot was chromatographed on a PEI-cellulose thin layer plate (20 cm).

The thin layer was first developed with 1 M acetic acid, to a height of 5 cm, and then with 0.3 M LiCl, to a height of 15 cm (Randerath, 1965).

Preparation of (^{32}P) St-1 RF Hae III digest markers: St-1 RF I DNA was digested with restriction endonuclease Hae III in a reaction mixture that contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM NaCl, 4 mM dithiothreitol, 0.25 mg/ml bovine serum albumin and enzyme (1 unit/µg DNA) for 15 minutes at 37°C. The DNA was then dephosphorylated with E. coli alkaline phosphatase (0.006 units/µg DNA) for 60 minutes at 65°C. The enzyme was removed by phenol extraction and the DNA was precipitated with ethanol. The dephosphorylated DNA was labelled with polynucleotide kinase as previously described (Maxam and Gilbert, 1977). The DNA was first denatured by heating at 90°C for 2 minutes in 40 µl of 10 mM glycine-NaOH (pH 9.5), 1 mM spermidine and 0.1 mM EDTA. 5 µl of 0.5 M glycine-NaOH (pH 9.5), 0.1 M $MgCl_2$, 0.05 M dithiothreitol, 400 pmoles of (γ-^{32}P) ATP (2000-3000 Ci/mmole) and 4 units of T4 polynucleotide kinase was then added. After 15 minutes at 37°C the DNA was precipitated by the addition of 2 M ammonium acetate and three volumes of ethanol, reprecipitated from 0.3 M sodium acetate with three volumes of ethanol, rinsed with ethanol and dried.

Other procedures: All four deoxyribonucleoside triphosphates were purified free of ribonucleoside triphosphates by oxidation with sodium periodate and subsequent chromatography on DEAE-Sephadex as previously described (Wu, 1970).

Part one

Evidence that the dnaG protein catalyzes the synthesis of a ribo- or deoxyribonucleotide primer for DNA synthesis on G4 and øx174 single-stranded phage DNA.

Introduction

The initiation of øx174 replicative form (RF II) synthesis from the viral single-stranded DNA *in vitro* occurs in at least two distinct stages (Wickner and Hurwitz, 1974, 1975c, Ray, Capon and Gefter, 1976, Weiner, McMacken and Kornberg, 1976, McMacken, Ueda and Kornberg, 1977, Wickner, 1978). The first step ('prepriming') requires the activity of the *dnaB* and *dnaC* proteins, the DNA binding protein, and replication factors X, Y and Z (i, n, n' and n") to activate the DNA in an ATP-dependent reaction. This 'activated' DNA may be isolated as a complex by gel filtration chromatography after incubation of øx174 single-stranded DNA with ATP and either a partially purified cell extract (Ray, Capon and Gefter, 1976) or purified proteins (Wickner and Hurwitz, 1975c, Weiner, McMacken and Kornberg, 1976). In contrast to the kinetic lag observed for the conversion of unreacted øx174 DNA to RF II, 'activated' øx174 DNA supports immediate and rapid synthesis of the replicative form upon addition of the *dnaG* protein and components necessary for DNA chain elongation. The absolute requirement for the *dnaG* protein in this latter stage suggested that it plays an essential role in the initiation of DNA chain synthesis.

This idea was supported by the demonstration that phage G4 RF II synthesis requires only the *dnaG* protein and DNA binding protein in addition to DNA polymerase III and elongation factors (Zechel, Bouche and Kornberg, 1975, Bouche, Zechel and Kornberg, 1975). It was reported that the *dnaG* protein initiates DNA synthesis on G4 single-stranded DNA by synthesizing an oligoribonucleotide primer. In addition, it was suggested that the template for this reaction, G4 DNA complexed with DNA binding protein,

is the functional equivelant of the 'activated' øx174 DNA complex in that the dnaG protein catalyzes the same reaction on each DNA substrate (Weiner, McMacken and Kornberg, 1976).

As a means of characterizing the function of the dnaG protein, a thermolabile protein prepared from a dnaG^{ts} strain was utilized to separate the reaction catalyzed by the dnaG protein from subsequent DNA synthesis. The results of this approach confirm that the dnaG protein initiates DNA synthesis on G4 and 'activated' øx174 single-stranded DNA's by synthesizing a primer, and demonstrate that deoxyribonucleoside triphosphates as well as ribonucleoside triphosphates may serve as substrates in this reaction (Capon and Gefter, 1978). Similar findings have been reported by two other groups (Wickner, 1977, McMacken et al., 1977).

Results

As mentioned in the Introduction the nature of the requirement for the dnaG protein in the conversion of øx174 and G4 single-stranded DNA to RF II suggested a role for the protein in primer formation. The mechanism by which this occurs may be such that the dnaG protein acts in a stage that completely preceeds the onset of DNA synthesis, as for example, if it were to catalyze the synthesis of a polynucleotide primer as does RNA polymerase to initiate DNA synthesis on fd single-stranded DNA. Alternatively, priming by the dnaG protein would be tightly coupled to subsequent DNA synthesis if it were to occur via a 3' OH terminus generated by a protein-AMP intermediate bound to the template DNA. To distinguish between these possibilities a thermolabile dnaG protein, prepared from a dnaG^{ts} strain of E. coli, was utilized to determine whether the reaction catalyzed by the protein on G4 or 'activated' øx174 single-stranded DNA transpires prior to the onset of DNA synthesis.

As demonstrated in Table 1, DNA synthesis dependent upon either G4 or 'activated' øx174 single-stranded DNA requires the activity of the dnaG protein in addition to that of DNA polymerase III and elongation factors. The reaction with G4 DNA also requires the DNA binding protein as previously reported (Zechel, Bouche and Kornberg, 1975). Thus, either 'activated' øx174 DNA or G4 DNA complexed with DNA binding protein may be used to assay for the function of the dnaG protein.

The ability of the dnaG protein to prime DNA synthesis was examined in a two-stage reaction with the thermolabile protein as follows. The dnaG^{ts} protein was incubated along with either DNA and the other essential

Table 1 Requirements for synthesis of RF II from øx174 and G4 single-stranded DNA.

Condition	dTMP incorporation (pmoles/20 minutes) 'activated' øx174 DNA	G4 DNA
Complete	2.1	4.2
-dnaG protein	0.2	<0.1
-DNA polmerase III*, copolymerase III*	0.2	<0.1
-DNA binding protein	-	0.1
-'activated' øx174 DNA, +øx174 DNA	<0.1	-
-ATP	<0.1	0.3

Reaction mixtures (50 µl) contained reaction buffer (20 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 4 mM dithiothreitol, 2 mM KH_2PO_4, 30 µg/ml rifampicin, 0.5 mg/ml bovine serum albumin), either 0.1 nmole 'activated' øx174 DNA (prepared as described in Methods) plus 0.5 mM ATP or 0.05 nmole G4 DNA plus 0.1 µg DNA binding protein and 0.2 mM ATP, 0.04 mM each dATP, dGTP, dCTP and (^{3}H) dTTP (1000 cpm/pmole), 0.05 units dnaG protein, 0.1 units DNA polymerase III*, and 0.1 units copolymerase III*.

For two-stage reactions GTP, CTP and UTP are added at 0.1 mM where indicated.

protein components, but in the absence of deoxyribonucleoside triphosphates, at a temperature permissive for thermolabile protein function (30°C). Under these conditions DNA synthesis will not occur. The temperature is then raised to 38°C to inactivate the thermolabile protein and dNTP's are added, allowing DNA synthesis to begin only if a stable primer has been formed and the activity of the dnaG protein is no longer required.

As demonstrated in Table 2, although the addition of ATP to a control reaction, incubated throughout at the permissive temperature, is sufficient to support extensive DNA synthesis upon G4 single-stranded DNA (line 4), when ATP is added to a reaction first incubated at 30°C, but then raised to 38°C to inactivate the dnaG^{ts} protein before dNTP's are added, there is no ensuing DNA synthesis (line 5). In contrast, when the same experiment is performed with a mixture of all four ribonucleoside triphosphates, 82% as much DNA synthesis occurs after a temperature shift-up (line 2) as when the reaction is incubated at 30°C throughout (line 1), suggesting that a stable primer had been formed under these conditions prior to the inactivation of the dnaG^{ts} protein. These results provide strong confirmation for the previous finding that the dnaG protein is able to catalyze the synthesis of a ribonucleotide primer in a rifampicin-resistant reaction that initiates G4 replicative form synthesis (Bouche, Zechel and Kornberg, 1975), and further suggest that ATP alone is not sufficient for this reaction to occur. By examining other reactions in which either GTP alone (line 8) or ATP and GTP (line 6) are added, it is seen that the addition of ATP and GTP prior to the inactivation of the dnaG^{ts} protein is both necessary and sufficient to satisfy the ribonucleotide substrate requirement

Table 2 Ribonucleoside or deoxyribonucleoside triphosphates satisfy primer requirements for G4 DNA synthesis.

First stage additions (5 minutes @ 30°C)	Second stage additions 20 min @ 30°C	or 20 min @ 38°C	% reaction
1. ATP,GTP,CTP,UTP	4dNTP	-	100 (7 pmole)
2. ATP,GTP,CTP,UTP	-	4dNTP	82
3. ATP,GTP,CTP,UTP @ 38°C	-	4dNTP	<2
4. ATP	4dNTP	-	94
5. ATP	-	4dNTP	<2
6. ATP,GTP	-	4dNTP	72
7. ATP	-	GTP,4dNTP	2.7
8. GTP	-	4dNTP	<2
9. GTP	-	ATP,4dNTP	4.0
10. ATP,UTP	-	4dNTP	<2
11. ATP,CTP	-	4dNTP	<2
12. ATP,UTP,CTP	-	4dNTP	<2
13. ATP,dTTP,dCTP,dATP	-	dGTP	<2
14. ATP,dTTP,dGTP,dATP	-	dCTP	<2
15. ATP,dATP,dCTP,dGTP	-	dTTP	<2
16. ATP,dTTP,dGTP,dCTP	-	dATP	56
17. ATP,GTP,dTTP,dGTP,dCTP	-	dATP	117
18. ATP,dTTP,dGTP,dCTP	-	-	<2

In addition to nucleoside triphosphates as indicated first stage reaction mixtures contained G4 DNA, *dnaG*^{ts} protein, DNA binding protein, DNA polymerase III* and copolymerase III* (amounts given in Table 1).

for primer generation with G4 DNA. If either ATP (line 9) or GTP (line 7) is omitted from the permissive incubation and added to the reaction after the shift to 38°C, there is no subsequent DNA synthesis.

One question raised by these results concerns why GTP is required for primer formation in a two-stage reaction but is not ordinarily required in a reaction containing active dnaG protein throughout. One possibility is that deoxyribonucleotides, normally present in the reaction, may also serve as substrates for priming catalyzed by the dnaG protein. To investigate this question, two-stage reactions were carried out with a mixture of three deoxyribonucleotides and ATP present during the permissive incubation, with the fourth deoxyribonucleotide added after the shift to 38°C. Under these conditions there is no detectable DNA synthesis until the fourth deoxyribonucleotide is added (for example, line 18).

As seen on line 16, the addition of ATP, dTTP, dGTP and dCTP during the permissive incubation in a two-stage reaction leads to 56% as much DNA synthesis as occurs in a reaction carried out entirely at 30°C (line 4). There is no detectable DNA synthesis if either dTTP (line 15), dGTP (line 13) or dCTP (line 14) is omitted during the permissive incubation. Thus, dTTP, dGTP and dCTP together appear to replace GTP as substrates for the generation of a stable primer for G4 replicative form synthesis. When GTP is included in addition to ATP, dTTP, dGTP and dCTP, subsequent DNA synthesis is stimulated two-fold (line 17), suggesting that the concentration of substrates is limiting for priming under these conditions, and that the contribution of ribo- and deoxyribonucleotides is additive.

In contrast to the results observed for G4 replicative form synthesis, ribonucleoside triphosphates alone do not support the formation of a stable

primer by the dnaG protein with 'activated' øx174 single-stranded DNA. As shown in Table 3, although ATP is the only ribonucleoside triphosphate normally required for the synthesis of øx174 RF II in a reaction incubated entirely at 30°C (line 5), the presence of either ATP (line 6) or a mixture of all four rNTP's (line 2) during the permissive incubation in a two-stage reaction is not sufficient to promote priming by the thermolabile dnaG^{ts} protein. In each instance the addition of thermostable dnaG protein to the reaction after the shift to 38°C restores subsequent DNA synthesis to a normal level (lines 3 and 7), ruling out the possibility that the 'activated' øx174 DNA substrate is inactivated under these conditions. These results suggest that deoxyribonucleoside triphosphates are essential for priming by the dnaG protein with øx174 DNA.

This prediction is confimed by the results shown in Table 4. The addition of ATP, dTTP, dGTP and dCTP during the permissive incubation of a two-stage reaction leads to 45% as much DNA synthesis as observed for an identical reaction incubated entirely at 30°C (compare lines 8 and 9). There is no detectable DNA synthesis when dCTP is omitted from the permissive incubation and added back to the reaction after the shift to 38°C (line 5), suggesting that this nucleotide is absolutely required for priming. In contrast, when either dTTP (line 11) or dGTP (line 7) is omitted, subsequent DNA synthesis is reduced several-fold, suggesting that these nucleotides are not essential but stimulatory to priming.

Table 3 Ribonucleoside triphosphates do not satisfy the priming requirement for øx174 RF II synthesis from 'activated' øx174 single-stranded DNA.

First stage additions (5 minutes at 30°C)	Second stage additions: 20 min @ 30°C	or 20 min @ 38°C	% reaction
1. ATP,GTP,CTP,UTP	4 dNTP	-	100
2. ATP,GTP,CTP,UTP	-	4 dNTP	<4
3. ATP,GTP,CTP,UTP	-	4 dNTP, *dnaG*$^{+}$ protein	112
4. ATP,GTP,CTP,UTP 5 minutes @ 38°C	-	4 dNTP	<4
5. ATP	4 dNTP	-	100
6. ATP	-	4 dNTP	<4
7. ATP	-	4 dNTP, *dnaG*$^{+}$ protein	210
8. ATP 5 minutes @ 38°C	-	4 dNTP	<4

In addition to the indicated nucleoside triphosphates (0.5 mM ATP, 0.1 mM each GTP, CTP and UTP, 0.04 mM each dNTP) first stage reaction mixtures contained 'activated' øx174 DNA, *dnaG*^{ts} protein, DNA polymerase III*, and copolymerase III* (concentrations given in Table 1).

Table 4 Deoxyribonucleoside triphosphates and ATP satisfy priming requirement for øx174 RF II synthesis from 'activated' øx174 DNA.

First stage additions (5 minutes @ 30°C)	Second stage additions 20 min @ 30°C	or 20 min @ 38°C	% reaction
1. ATP	4dNTP	-	100
2. ATP	-	4dNTP	<5
3. ATP (5 minutes @ 38°C)	-	4dNTP	<5
4. ATP,TTP,dATP,dGTP	dCTP	-	72
5. ATP,TTP,dATP,dGTP	-	dCTP	<5
6. ATP,TTP,dATP,dCTP	dGTP	-	79
7. ATP,TTP,dATP,dCTP	-	dGTP	13
8. ATP,TTP,dCTP,dGTP	dATP	-	100
9. ATP,TTP,dCTP,dGTP	-	dATP	45
10. ATP,dATP,dCTP,dGTP	TTP	-	100
11. ATP,dATP,dCTP,dGTP	-	TTP	13

Reaction mixtures were as described in Table 3.

Discussion

The use of a thermolabile protein, prepared from a *dnaG*^{ts} strain of *E. coli*, has demonstrated conclusively that the ability to prime synthesis of RF II on either øx174 or G4 single-stranded DNA resides with the *dnaG* protein. In confirmation of earlier studies, ribonucleoside triphosphates were found to serve as substrates for priming on G4 DNA (Bouche, Zechel Kornberg, 1975). In the absence of deoxyribonucleoside triphosphates the addition of ATP and GTP is both sufficient and necessary to observe efficient priming. In addition, the experiments described here demonstrate that dNTP's may also serve as substrates for priming on G4 DNA and are essential for priming on øx174 DNA. These findings are in agreement with reports by other investigators which show that the *dnaG* protein catalyzes the synthesis of oligonucleotides, comprised of both ribo- and deoxyribonucleotides, which become covalently attached to the DNA product synthesized with either G4 DNA (Wickner, 1977, Rowen and Kornberg, 1978) or øx174 DNA (McMacken and Kornberg, 1978).

In contrast to this study, it has been reported that the *dnaG* protein may catalyze priming without deoxyribonucleotides on 'activated' øx174 DNA that has been prepared with purified proteins: DNA binding protein, the *dnaB* and *dnaC* proteins, and factors i and n (McMacken, Ueda and Kornberg, 1977). One difference between these studies is that the 'activated' øx174 DNA substrate utilized here was isolated by gel filtration chromatography after incubating the DNA with a crude cell extract and may therefore contain enzymatic activities that preferentially destroy primers consisting mainly of ribonucleotides. One such activity that preferentially degrades

RNA:DNA hybrids is Ribonuclease H, which has been shown to be part of a system operating in crude extracts that selectively hydrolyzes ribonucleotide primers synthesisized by RNA polymerase on øx174 DNA, and not fd DNA, in the presence of DNA binding protein and two other proteins, called discriminatory factors α and β (Vicuna et al., 1977, Vicuna, Ikeda and Hurwitz, 1977).

These observations are relevant in light of the uncertainty concerning the covalent attachment of RNA to the 5' end of nascent DNA chains in E. coli (see General Introduction). As demonstrated in Part two, the dnaG protein is capable of efficiently synthesizing a primer with deoxyribonucleotides exclusively (on St-1 single-stranded DNA). This results raise the possibility that DNA rather than RNA primers serve to initiate the synthesis of DNA chains in E. coli.

Part two

The mechanism of primer synthesis catalyzed by the dnaG protein.

Introduction

As demonstrated in Part one, and by other investigators, the dnaG protein of E. coli initiates the conversion of phage øx174 and G4 single-stranded DNA to the duplex replicative form (RF II) in vitro by catalyzing the synthesis of a primer, utilizing both ribo- and deoxyribonucleoside triphosphates as substrates (Wickner, 1977, McMacken et al., 1977, Capon and Gefter, 1978). The participation of auxiliary proteins distinguishes the mechanism of priming on either DNA template. øx174 DNA must first be 'activated' in an ATP-dependent reaction that requires the DNA binding protein, the dnaB and dnaC proteins, and replication factors X, Y and Z (i, n, n' and n") before the dnaG protein can act (Wickner and Hurwitz, 1975c, Weiner, McMacken and Kornberg, 1976, Ray, Capon and Gefter, 1976). In contrast, a much simpler interaction requiring only the DNA binding protein is sufficient to create a 'site' at which the dnaG protein may prime DNA synthesis on G4 and St-1 single-stranded DNA (Zechel, Bouche and Kornberg, 1975, Wickner, 1977). One consequence of this difference may be that while the synthesis of RF II appears to be initiated at many sites on øx174 DNA (Eisenberg et al., 1975, McMacken, Ueda and Kornberg, 1977), initiation occurs at a single location on G4 DNA (Bouche, Zechel and Kornberg, 1975, Hourcade and Dressler, 1978).

A role for the dnaG protein in the initiation of DNA chain synthesis in E. coli has been suggested by in vivo studies (Lark, 1972a, Louarn, 1974). To illuminate this process, the mechanism of primer synthesis by the dnaG protein has been investigated in greater detail. Attention has been focused on the simpler DNA templates, such as G4 and St-1, which direct the synthesis

of a unique primer, to provide a framework for examining the influence of ribo- vs. deoxyribonucleotide substrates on the initiation, continuation and completion of primer synthesis. These studies demonstrate that the dnaG protein utilizes deoxyribonucleotides as efficiently as ribonucleotides to catalyze primer synthesis on St-1 single-stranded DNA, although ATP is preferred to dATP as the initiating nucleotide. The possibility that DNA chain synthesis in E. coli is initiated with DNA rather than RNA priming, is thus suggested.

Priming by the dnaG protein in the presence of DNA binding protein, as well as binding of the dnaG protein to DNA, is specific for G4 and St-1 DNA, but not fd or øx174 DNA (Wickner, 1977). In order to characterize the nature of this specific interaction, other single-stranded DNA phages, for which the synthesis of RF is initiated by the same pathway as G4 and St-1, were identified and studied. It is shown here that the initiation of RF II synthesis from øK and α3 single-stranded DNA in vitro requires the dnaG protein and DNA binding protein only. The oligonucleotide primers synthesized with St-1, øK and α3 DNA are each unique and have an identical nucleotide sequence. This sequence is very similar to that reported for the primer synthesized on G4 DNA (Bouche, Rowen and Kornberg, 1978, D. Capon and S. Wickner, unpublished observations), suggesting that specificity is dictated by a highly conserved template recognition element. These studies were extended by locating the sites on St-1, øK and α3 DNA at which synthesis of the complementary strand is initiated in vitro and determining the nucleotide sequence of this region (Sims, Capon and Dressler, 1979). A highly conserved intercistronic region of 135 nucleotides containing a sequence complementary to that of the primer synthesized by the dnaG protein was thus

identified on each phage DNA. This region exhibits the potential for forming two stable hairpin structures, one of which is transcribed by the dnaG protein.

Primer synthesis on St-1, øK and α3 DNA has been shown to be initiated at a unique site with ATP or dATP, and to extend for 10 to 28 nucleotides in the absence of DNA synthesis. In addition, these studies demonstrate that there is a kinetic barrier to the formation of primers longer than 11 nucleotides. This barrier may be overcome by increasing the substrate concentration or the length of incubation, and is influenced by conditions that affect template secondary structure such as salt concentration and temperature. Synthesis of primers longer than 28 nucleotides has not been observed under any conditions.

To determine the nature of the oligonucleotide that actually serves to prime DNA synthesis on St-1 single-stranded DNA, the nascent DNA product synthesized in vitro was examined for ribonucleotides covalently attached to its 5' end. Greater than 90% of the product was initiated from primers at least 9 to 11 nucleotides long. Although primer synthesis is completed earlier in the presence than absence of DNA synthesis, this effect is the same when DNA elongation is catalyzed by either DNA polymerase III with the dnaZ protein and elongation factors I and III or DNA polymerase I. These results suggests that the observed coupling effect is due to the dissociation of the dnaG protein from the template rather than a physical interaction between the dnaG protein and a DNA polymerase.

Results

Requirements for DNA synthesis on single-stranded phage DNA primed by the dnaG protein

Previous studies have shown that in the presence of the E. coli DNA binding protein, the dnaG protein catalyzes priming on G4 and St-1 but not fd or øx174 single-stranded DNA (Wickner, 1977, Rowen and Kornberg, 1978a). Other DNA templates which support the initiation of DNA synthesis by this mechanism were identified and characterized in detail to determine the basis for this specificity. In vivo studies had suggested that the synthesis of parental RF from infecting øK and α3 single-stranded phage DNA proceeds by the same pathway, requiring the dnaG protein, but not the dnaB and dnaC proteins (Taketo, 1976, Taketo and Kodaira, 1977).

The requirements for conversion of G4, St-1, øK and α3 single-stranded DNA to the duplex replicative form catalyzed by E. coli DNA polymerase I are shown in Table 5. Initiation of RF synthesis on G4 and St-1 DNA strictly requires the dnaG protein and DNA binding protein as previously reported (Bouche, Zechel and Kornberg, 1975, Wickner, 1977). The addition of the dnaG protein and DNA binding protein is also necessary and sufficient to observe efficient DNA synthesis on both øK and α3 single-stranded DNA. As demonstrated later, similar results were obtained when DNA synthesis was catalyzed by DNA polymerase III and elongation factors instead of DNA polymerase I. The product of these reactions was full-length linear complementary strand DNA as determined by velocity sedimentation in an alkaline sucrose gradient with circular (^{14}C) øx174 single-stranded DNA

Table 5 Requirements for St-1, øK, α3 and G4 DNA-dependent DNA synthesis.

	dTMP incorporation (pmoles/20 minutes)			
Condition	St-1 DNA	øK DNA	α3 DNA	G4 DNA
Complete	15.5	42.1	24.6	15.4
-*dnaG* protein	0.2	1.9	1.6	0.6
-DNA binding protein	0.6	2.2	1.6	1.5
-ATP	9.8	10.5	N.D.	2.1
-DNA polymerase I	<0.1	<0.1	<0.1	<0.1

Reaction mixtures (25 µl) contained 0.4 nmoles (as nucleotide) DNA, 0.04 units *dnaG* protein, 0.35 µg DNA binding protein, 0.3 units DNA polymerase I, 0.2 mM ATP, 0.04 mM each dATP, dGTP, dCTP and (^{3}H) dTTP (1000 cpm/pmole), and reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 4 mM dithiothreitol, 0.5 mg/ml bovine serum albumin and 20 µg/ml rifampicin). After 20 minutes at 30°C, reactions were terminated and dTMP incorporation determined as described in Methods.

N.D., not done.

as marker (data not shown).

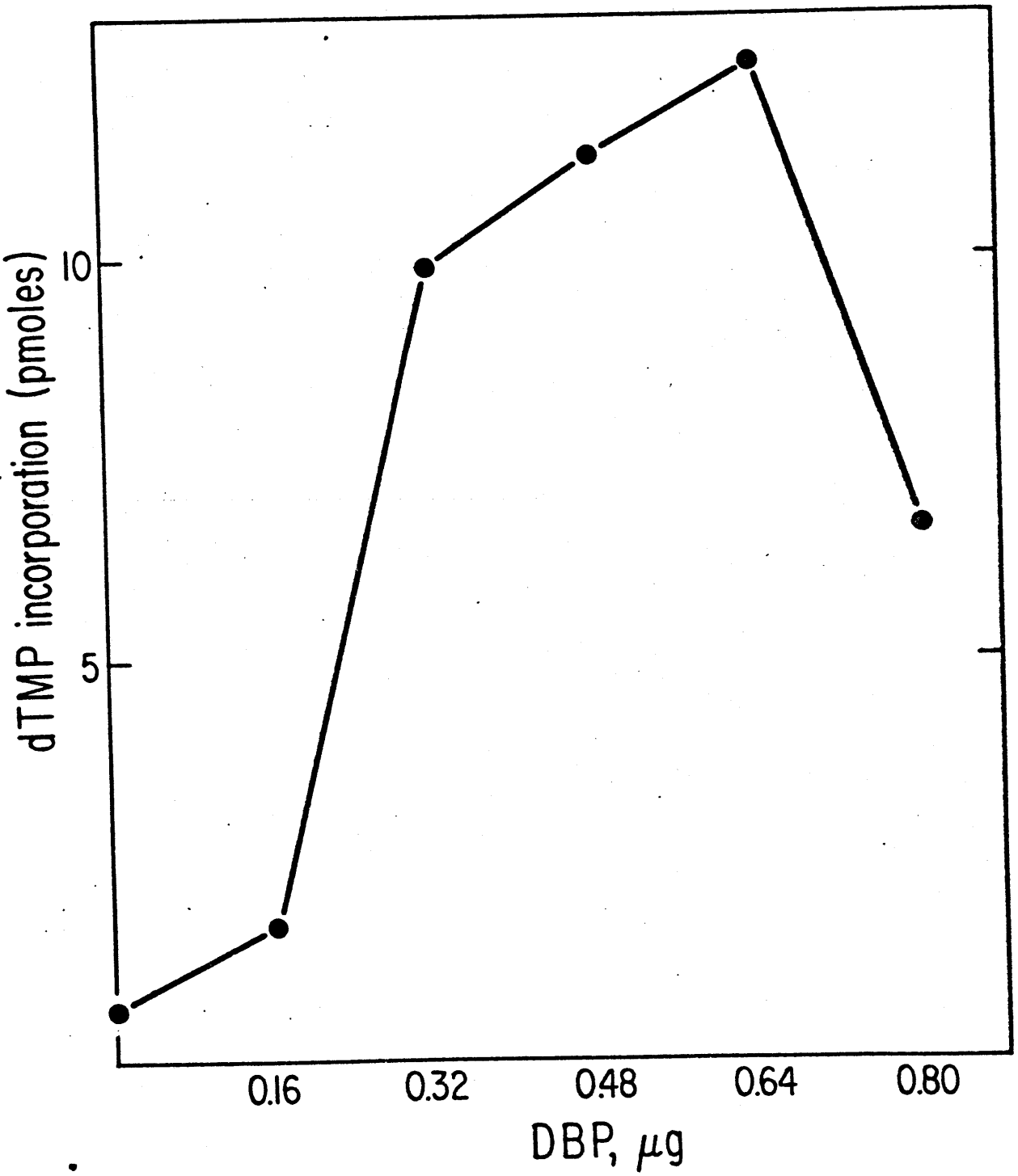
DNA synthesis was stimulated by the addition of ATP to the reaction to an extent that depended on the particular DNA template employed. This stimulation was 7.5-fold, 4-fold and 1.5-fold with G4, øK and St-1 DNA, respectively. This difference may reflect the ability of dATP to substitute for ATP as a substrate for dnaG catalyzed primer synthesis. As shown later, dATP efficiently replaces ATP for primer synthesis on St-1 DNA.

Half-maximal stimulation by ATP was observed at a concentration of 50 μM for G4 DNA synthesis and 25 μM for øK DNA synthesis. GTP, CTP and UTP were not stimulatory with any of the four DNA templates at optimal concentrations of the other reaction components. As previously reported, ADP stimulated DNA synthesis to the same maximal extent as ATP, but at lower concentrations (Wickner, 1977). Half-maximal stimulation of DNA synthesis by ADP occurred at 10 μM with G4 DNA and 5 μM with øK DNA.

The extent of DNA synthesis was linearly proportional to the amount of dnaG protein added to the reaction. In contrast, the extent of DNA synthesis observed with St-1 DNA exhibits a higher-order dependence upon the DNA binding protein concentration, as shown in Figure 1. The DNA binding protein concentration at which DNA synthesis is maximally stimulated is sufficient to saturate only 1/4 to 1/2 of DNA template (DNA binding protein: St-1 DNA, 2.4-4.8:1, w/w) assuming that each DNA binding protein monomer binds approximately 8 nucleotides of single-stranded DNA as previously demonstrated (Sigal et al., Molineux, Pauli and Gefter, 1975). Further increases in the DNA binding protein concentration are inhibitory.

Figure 1 Extent of St-1 DNA-dependent DNA synthesis at different DNA binding protein concentration.

Reaction mixtures (25 µl) contained 0.4 nmoles of St-1 DNA, 0.04 units of dnaG protein, 0.3 units of DNA polymerase I and the indicated amount of DNA binding protein, and were incubated at 30°C for 20 minutes. The addition of 0.48 µg of DNA binding protein corresponds to a weight ratio for binding protein:DNA of 3.6:1 or approximately 42% saturation of the template, assuming 100% saturation at a ratio of 8.5:1 (Molineux, Pauli and Gefter, 1975).


dTMP incorporation (pmoles)
10
5
0.16
0.32
0.48
0.64
0.80
DBP, μg

Characteristics of oligoribonucleotide primer synthesis catalyzed by the dnaG protein on St-1, øK and α3 single-stranded DNA.

As demonstrated in Part one, the priming step catalyzed by the dnaG protein on øx174 and G4 single-stranded DNA may be separated from subsequent DNA synthesis. The product synthesized in this partial reaction with G4 or St-1 DNA has been characterized as an oligonucleotide primer, comprised either of ribonucleotides (Bouche, Zechel and Kornberg, 1975) or of both ribo- and deoxyribonucleotides (Wickner, 1977, Rowen and Kornberg, 1978b). These studies have shown that oligonucleotide synthesis is resistant to rifampicin and requires the DNA binding protein, ATP or ADP, and ribo- or deoxyribonucleoside triphosphates in addition to the dnaG protein and G4 or St-1 single-stranded DNA. The product remains associated with the DNA template after its synthesis, and on this basis may be purified away from unreacted substrate by gel filtration chromatography. This oligonucleotide-DNA complex supports extensive DNA synthesis upon addition of DNA polymerase and elongation factors.

The detailed mechanism by which the dnaG protein initiates and completes the synthesis of primers on St-1, øK and α3 single-stranded DNA has been investigated. The principal questions addressed in this study concern the nucleotide sequence and composition of the products, conditions which affect the length of the products, the nature of the template site that promotes primer synthesis, the role of ribo- and deoxyribonucleotides in primer synthesis, and the nature of the primer that is synthesized when priming is coupled to DNA synthesis. To facilitate the determination of nucleotide sequences, the characteristics of primer synthesis in reactions containing

ribonucleotide substrates only, were investigated first.

As expected from the ability of the dnaG protein to efficiently initiate DNA synthesis on St-1, øK and α3 single-stranded DNA (Table 5), the dnaG protein also catalyzed the incorporation of rNTP's into an oligoribonucleotide product with these same templates, which remains associated with the DNA after its synthesis and may be isolated by Sepharose 6B gel filtration (Table 6). Oligoribonucleotide synthesis absolutely requires the dnaG protein and DNA binding protein, and is specific for St-1, øK, α3 and G4 single-stranded DNA, but not øx174 single-stranded DNA (Table 6). 1 pmole of St-1, øK and α3 DNA molecules supported the incorporation of 13.6, 15.7 and 15.4 pmoles of total ribonucleotide into product, respectively. Since most of the product was 22-26 nucleotides in length under these conditions (data not shown), this extent of oligoribonucleotide synthesis represents priming of more than half of the imput DNA.

The product synthesized by the dnaG protein on øK single-stranded DNA in the presence of all four rNTP's is a heterogeneous collection of oligoribonucleotides, 10 to 28 nucleotides in length, as determined by electrophoresis on a denaturing polyacrylamide gel (Figure 2). Most of the product is found in one of two predominant size classes that are 11 and 22-26 nucleotides long, respectively. At a given rNTP concentration, the pattern of products synthesized with St-1 or α3 DNA was very similar to that synthesized with øK DNA (see below). Of particular interest is the observation that all of the product appears as discrete bands on the gel at intervals that are spaced regularly (Figure 2). This suggests that all of the oligonucleotides synthesized in a particular reaction share the same nucleotide sequence but differ by the sites at which their synthesis

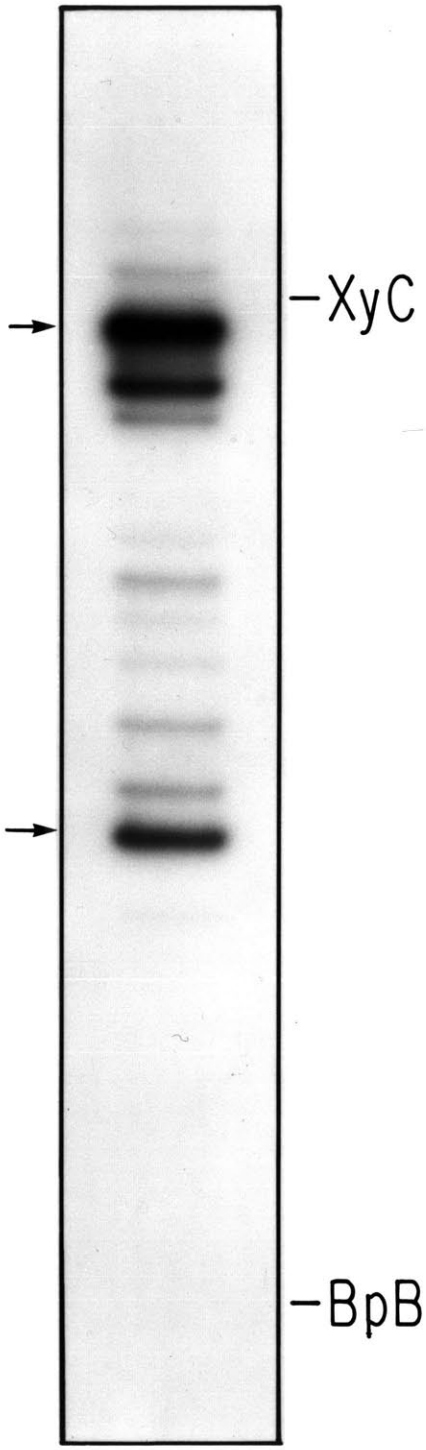
Table 6 Requirements for oligoribonucleotide synthesis by the dnaG protein.

	Total NMP incorporation (pmoles/20 minutes)				
Condition	St-1 DNA	øK DNA	α3 DNA	G4 DNA	øx174 DNA
Complete	10.2	11.8	11.6	7.7	<0.01
-DNA	<0.01				
-dnaG protein	<0.01				
-DNA binding protein	<0.01				

Reaction mixtures (25 µl) contained 4.5 nmoles (as nucleotide) DNA, 1.1 units dnaG protein, 3.3 µg DNA binding protein, and 75 mM each (α-^{32}P) ATP, GTP, CTP and UTP (35 Ci/mmole), and were performed as described in the legend to Figure 2. 4.5 nmoles (as nucleotide) of St-1 single-stranded DNA corresponds to 0.75 pmoles of St-1 DNA molecules, assuming a length of 6050 nucleotides (Grindley and Godson, 1978).

Figure 2 øK DNA-dependent oligoribonucleotide synthesis catalyzed by the dnaG protein.

Reaction products were electrophoresed on a 20% polyacrylamide gel in the presence of 7 M urea (anode at bottom). Reaction mixture (50 µl) contained 8.6 nmoles (as nucleotide) øK DNA, 2.4 units dnaG protein, 5.6 µg DNA binding protein, 25 µM each (α-^{32}P) ATP, GTP, CTP and UTP (200-300 Ci/mmole) and reaction buffer (50 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin and 20 µg/ml rifampicin). After 20-30 minutes at 30°C, the product was purified away from unreacted substrate by Sepharose 6B chromatography as descibed in Methods. XyC and BpB denote the positions of the marker dyes xylene cyanol and bromophenol blue, respectively. The arrows on the left indicate the longer (22-26 nucleotides) and shorter (11 nucleotides) products that were analyzed by the T_1 fingerprinting method as described in the text.


XyC
BpB

is either initiated or completed.

Nucleotide sequence of the St-1 oligoribonucleotide products

In order to learn the exact relationship between the primers synthesized on a particular DNA template, as well as the similarities between primers synthesized on different templates, the nucleotide sequences of the products synthesized by the dnaG protein on St-1, ØK and α3 DNA were determined. The approach initially taken was to analyze uniformly radioactive oligoribonucleotide products synthesized in reactions containing all four (α-^{32}P) ribonucleoside triphosphates. Representatives of the long (23-26 nucleotides) and short (11 nucleotides) classes of product, indicated by the arrows in Figure 2, were purified on preparative denaturing polyacrylamide gels. The total, long and short classes of product were individually hydrolysed with ribonuclease T_1, and the products of each digestion were fractionated by the two-dimensional 'fingerprinting' method (Sanger, Brownlee and Barrell, 1965).

The results of this analysis for the long and short classes of oligoribonucleotide synthesized with St-1 DNA are shown in Figures 3A and 3B, respectively, and depicted in schematic form in Figure 4 for reference. The simplicity of each pattern of T_1 digestion products, 7 species for the long class and 5 species for the short class, suggests that each class of product comprises a single nucleotide sequence. Moreover, the similarity between the two fingerprint patterns implies that the sequence of the shorter product is entirely overlapped by that of the longer product. Nucleotide composition analysis, as follows, demonstrated that the corresponding T_1 digest nucleotides of each fingerprint (t1, t3, t4, t6 and t7)

Figure 3 T_1 ribonuclease fingerprints of the St-1 oligoribonucleotide products.

Uniformly radioactive oligoribonucleotide products were prepared in reactions containing St-1 DNA and separated according to chain length by gel electrophoresis as described in the legend to Figure 2. Panels (a) and (b) represent the long (24-26 nucleotides) and short (11 nucleotides) products, respectively, indicated by the arrows in Figure 2, which were individually eluted from the gel, digested with T_1 ribonuclease and subjected to two-dimensional electrophoresis (directions indicated by arrows) as described in Methods. Y and B represent the positions of the dye markers methyls orange and xylene cyanol, respectively.

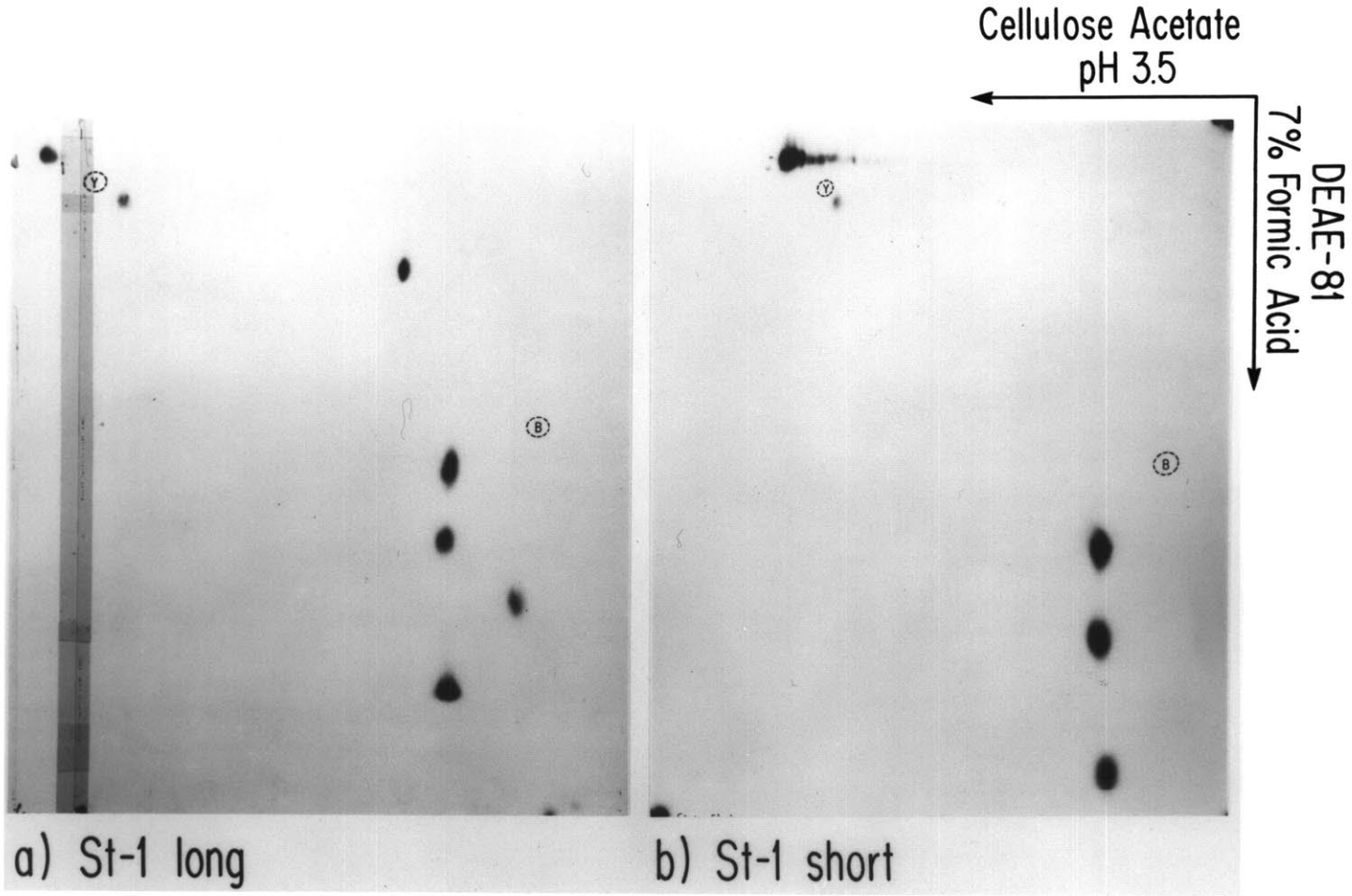
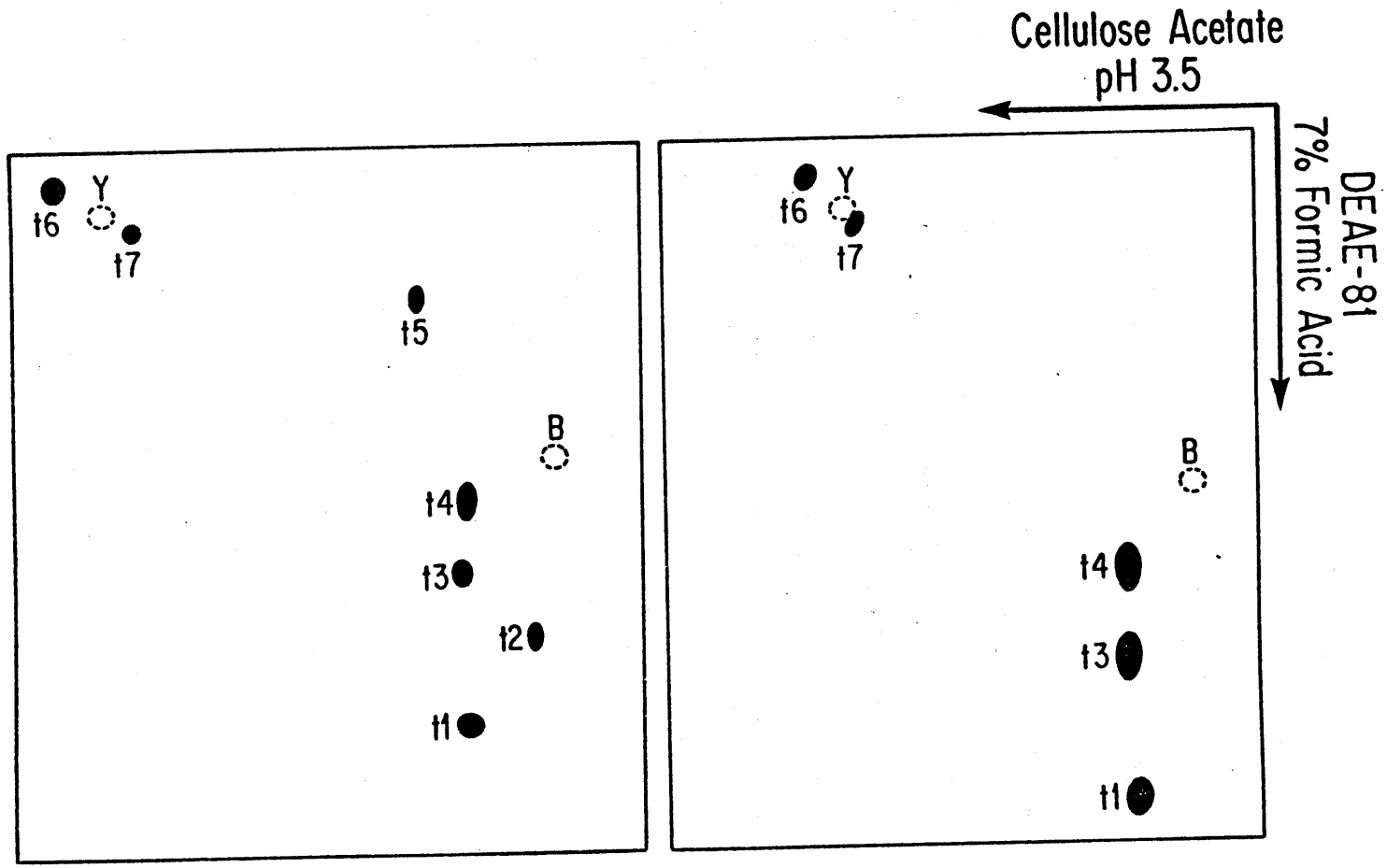

Cellulose Acetate
pH 3.5
DEAE-81
7% Formic Acid
Y
B
Y
B
a) St-1 long
b) St-1 short

Figure 4 Schematic representation of the T_1 ribonuclease fingerprints for the St-1, ØK and α3 oligoribonucleotide products.

Designations used for each T_1 ribonuclease digest nucleotide correspond to those used to summarize molar yields in Table 7.



a) St-1 long

b) St-1 short

are indeed identical. t2 and t5 are those digest products contained within the long but not short oligoribonucleotides.

The nucleotides Gp (t1), CpGp (t2), ApGp (t3) and ApApGp (t4) were identified by (1) their characteristic position on the fingerprint (Sanger and Brownlee, 1967), (2) alkaline hydrolysis followed by electrophoresis at pH 3.5 on Whatman 150 paper, and (3) susceptibility of t2 but not the others to pancreatic ribonuclease A.

Nucleotide t5 was digested with ribonuclease A and the products were separated by electrophoresis at pH 3.5 on DEAE-81 paper. This treatment yielded equal molar amounts of Gp, Cp, Up and a nucleotide with a mobility characteristic of either ApApCp or ApApUp (between ApGp and ApApGp) (Barrell, 1971). Determination of its composition by alkaline hydrolysis confirmed that this pancreatic ribonucleotide was ApApCp. Thus, t5 is a hexanucleotide of the composition (Cp, Up, ApApCp)Gp and was not characterized further.

Nucleotides t6 and t7 streaked and migrated slower in the first dimension of the T_1 fingerprint if 1 mM EDTA was not added to the electrophoresis buffer. This is characteristic of nucleotides with a triphosphate end such as pppGp (Brownlee, 1972). Alkaline hydrolysis of each nucleotide followed by electrophoresis at pH 3.5 on either Whatman 540 or DEAE-81 paper yielded Gp and a nucleotide with a mobility comparable to ppppA for t6 and pppA for t7. Treatment of either t6 or t7 with E. coli alkaline phosphatase released inorganic phosphate and a nucleotide identified as ApG by electrophoresis on DEAE-81 at both pH 1.9 and pH 3.5. These results are consistent with a structure of pppApGp and ppApGp for t6 and t7, respectively.

The molar yields of each T_1 nucleotide found in the digests of the long and short St-1 oligoribonucleotides are summarized in Table 7. For every mole of ApGp, ApApGp and CpGp (long product only) found, 4 moles of Gp were obtained for the long product and 3 moles of Gp for the short. Only 0.54 molar equivalents of t5 were found possibly due to poor transfer of this larger nucleotide from the cellulose-acetate strip. Generally, the total amount of radioactivity that occurred in t6 and t7 represented one molar equivelant . However, the relative proportion of these two 5' terminal nucleotides present in a particular T_1 digest varied from one experiment to the next.

Additional information was necessary to determine the precise sequence for both the long and short St-1 oligoribonucleotide products. To this end, uniformly radioactive short St-1 oligoribonucleotide was analyzed by the 'wandering spot' method (Sanger et al., 1973, Jay et al., 1974). After dephosphorylation with E. coli alkaline phosphatase to remove the 5' terminal heterogeneity, the oligoribonucleotide was digested stepwise from the 3' end with snake venom phosphodiesterase, and the resulting products were fractionated by a two-dimensional procedure as shown in Figure 5. This method separates the partial digest products on the basis of nucleotide composition in the first dimension (cellulose-acetate at pH 3.5) and chain length in the second dimension (homochromatography).

The two types of mobility shift that are observed between successive partial digest products of the short St-1 oligoribonucleotide are consistent with those expected from the removal of either AMP or GMP residues from the 3' end (assuming that the oligonucleotide does not contain any pyrimidine residues as suggested by the fingerprinting results). This information

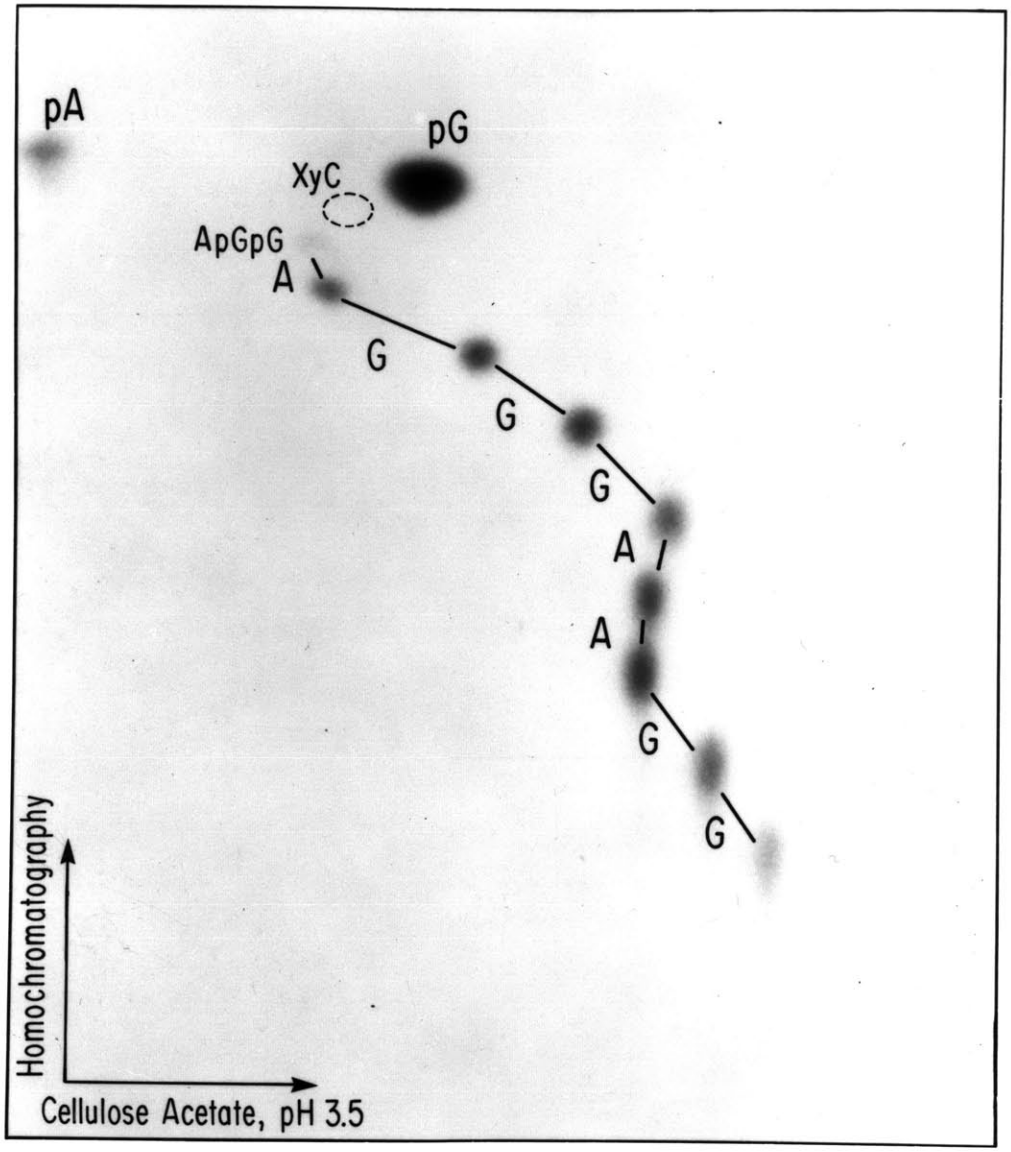
Table 7 Molar yields of the T_1 ribonuclease digest products of the St-1, øK and α3 oligoribonucleotide products synthesized by the dnaG protein.

		. . . . . Molar yields							
		calc. from structure*		St-1		øK		α3	
Spot no.	Sequence	Long	Short	Long	Short	Long	Short	Long	Short
t1	Gp	5	3	3.8	3.0	5.3	3.2	4.8	3.2
t2	CpGp	1	-	0.87	-	1.2	-	1.0	-
t3	ApGp	1	1	1.0	1.0	1.0	1.0	1.0	1.0
t4	ApApGp	1	1	1.2	0.89	1.2	0.86	1.1	0.85
t5	CpUpApApCpGp	1	-	0.54	-	0.50	-	0.40	-
t6	pppApGp			0.76	1.01	0.53	0.21	0.36	0.09
t7	ppApGp			0.28	0.11	0.44	0.40	0.58	0.70
t6 + t7		1	1	1.0	1.1	0.97	0.61	0.94	0.79

* Structure of the øK and α3 oligoribonucleotides is predicted from the nucleotide sequence of the øK and α3 DNA replication origins (Sims, Capon and Dressler, 1979).

Figure 5 'Wandering spot' sequence determination for the short St-1 oligoribonucleotide.

Uniformly radioactive St-1 short oligoribonucleotide prepared as described in the legend to Figure 10 was dephosphorylated with E. coli alkaline phosphatase to remove 5' terminal heterogeneity, partially digested with snake venom phosphodiesterase and analysed by a two-dimensional separation procedure. In the first dimension electrophoresis on cellulose acetate at pH 3.5 separates the digest products according to composition. Removal of an A or G residue results in a small increase and large decrease, respectively, in the mobility of the resulting oligonucleotide product. 5'-AMP and GMP residues removed stepwise from the 3' end of the oligonucleotide appear at the top of the autoradiograph. In the second dimension homochromatography on DEAE thin layers at pH 4.7 separates the digest products according to chain length, with shorter species migrating faster. The digestion conditions used here produced a collection of products 3 to 11 nucleotides in length. Figure 6 presents an analysis of the products of a longer digestion which are 2 to 5 nucleotides in length.


pA
pG
XyC
ApGpG
A
G
G
G
A
A
G
G
Homochromatography
Cellulose Acetate, pH 3.5

permits a direct reading of the 3' portion of sequence of the short St-1 oligoribonucleotide as -A-G-G-G-A-A-G-G_{OH}.

The 5' portion of the sequence was established by identifying the smallest partial digest products, which were resolved by electrophoresis on DEAE-81 paper at pH 1.9 as shown in Figure 6. ApG and ApGpG were identified by comparison with marker nucleotides . The identities of ApGpGpA and ApGpGpApG were confirmed by digesting these products with T_1 ribonuclease and electrophoresing the resulting digest on DEAE-81 at pH 3.5 (data not shown). Thus, the 5' portion of the sequence was (p)ppA-G-G-A-G-.

This additional information is sufficient to establish a sequence for the short St-1 oligoribonucleotide that is entirely consistent with the T_1 fingerprinting results,

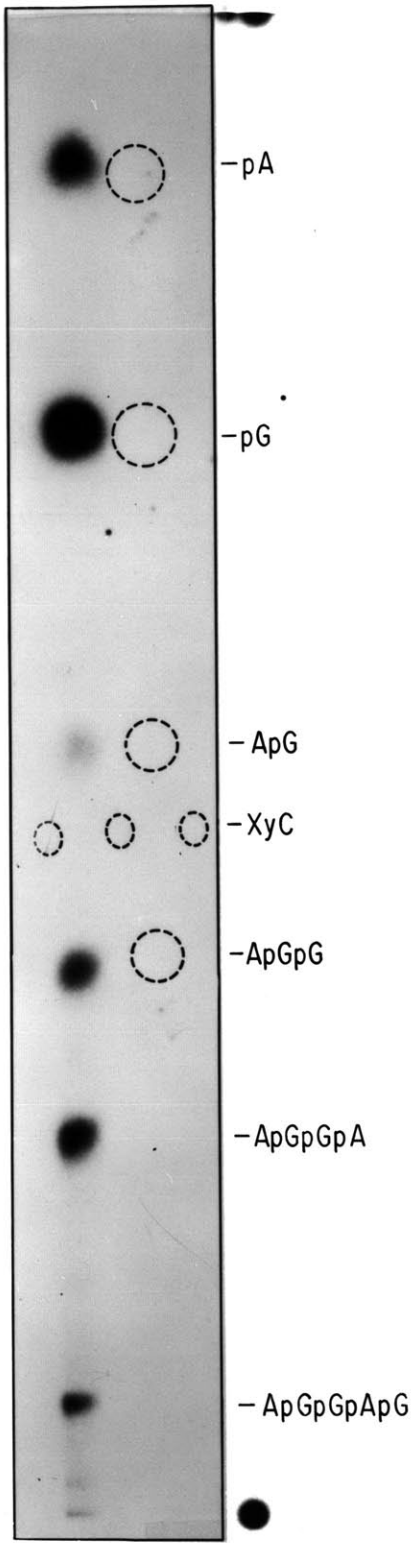
5' (p)ppA-G-G-A-G-G-G-A-A-G-G_{OH} 3'.

To facilitate sequence analysis of the long St-1 oligoribonucleotide, it was first demonstrated that only ATP (or ADP) may be incorporated at the 5' terminal position. The total St-1 oligoribonucleotide product was hydrolyzed with either alkali or a mixture of ribonucleases A, T_1 and T_2 and electrophoresed on DEAE-81 at pH 3.5. This treatment yielded only pppAp and ppAp in addition to the four 3' rNTP's (identified as described for the analysis of t6 and t7). The structure of these nucleotides was confirmed by digesting each with a large excess of snake venom phosphodiesterase (Brownlee, 1972). The resulting product was pAp as determined by electrophoresis at pH 3.5 on Whatman 540 paper (data not shown).

That only ATP (or ADP) is incorporated at the initiating position of the St-1 oligoribonucleotide products made it possible to radioactively

Figure 6 Analysis of the 5' terminal sequence for the short St-1 oligoribonucleotide.

Partial digest products of the short St-1 oligoribonucleotide, 2 to 5 nucleotides in length, were prepared in longer digestions with snake venom phosphodiesterase as otherwise described in the legend to Figure 5 and electrophoresed on DEAE-81 paper at pH 1.9. The identity of each product was confirmed by (1) comparison with the non-radioactive marker nucleotides ApG and ApGpG (kindly provided by Dr. H.G. Khorana) indicated by dotted circles in the adjacent lane, and (2) T_1 ribonuclease digestion followed by re-electrophoresis upon DEAE-81 paper at 3.5.


–pA
–pG
– ApG
–XyC
–ApGpG
–ApGpGpA
– ApGpGpApG

label these products by the direct incorporation of (γ-^{32}P) ATP during synthesis. Long St-1 oligoribonucleotide prepared in this fashion was sequenced by a method which compares the cleavage patterns obtained by partially digesting the product with various ribonucleases and resolving the digest products according to chain length by electrophoresis on a denaturing polyacrylamide gel (Donis-Keller, Maxam and Gilbert, 1977, Lockard et al., 1978) as shown in Figure 7.

Comparison of the partial digests produced by alkali, which cleaves after every residue, with those produced by T_1 ribonuclease reveals GMP residues at positions 2, 3, 5, 6, 7, 10, 11, 13 and 14 of the long St-1 oligoribonucleotide. The positions of AMP, CMP and UMP residues were determined by comparing the partial digest patterns obtained with ribonuclease U_2, which cleaves after AMP residues, B. cereus ribonuclease, which cleaves after pyrimidine residues, and Physarym I ribonuclease, which cleaves after UMP residues, and AMP residues followed by a purine or UMP residue. These positions are as follows (Figure 7): AMP- 1, 4, 8, 9 and 17; CMP- 12 and 15; UMP- 16.

To provide additional confirmation of these results, particularly at position 16 which was susceptible to cleavage by a contaminating nuclease, the 5' end-labeled long St-1 oligoribonucleotide was partially hydrolyzed with alkali and then analyzed by two-dimensional gel electrophoresis (Lockard et al., 1978). This method separates the digest products on the basis of composition in the first dimension and chain length in the second dimension. Mobility shifts between successive partial digest products that differ by a UMP or GMP residue may be distinguished from those differing by a CMP or AMP residue, which are more angular.

Figure 7 Partial ribonuclease digest products of 5' end-labeled long St-1 oligoribonucleotide separated by gel electrophoresis.

5' end-labeled long St-1 oligoribonucleotide was prepared as otherwise described in the legend to Figure 3 in a reaction which contained non-radioactive GTP,UTP and CTP and (γ-^{32}P) ATP (3000-4000 Ci/mmole) each at 25 µM. This product was partially digested with either alkali or ribonuclease (conditions described in Methods) as indicated and electrophoresed on a 20 % polyacrylamide gel containing 7 M urea (8 hours at 1,000 volts) to resolve the cleavage products according to chain length. The following amount of ribonuclease per 5 µg carrier tRNA was used in the digestions shown (left to right):

T_1, 0.1 units

T_1, 0.01 units

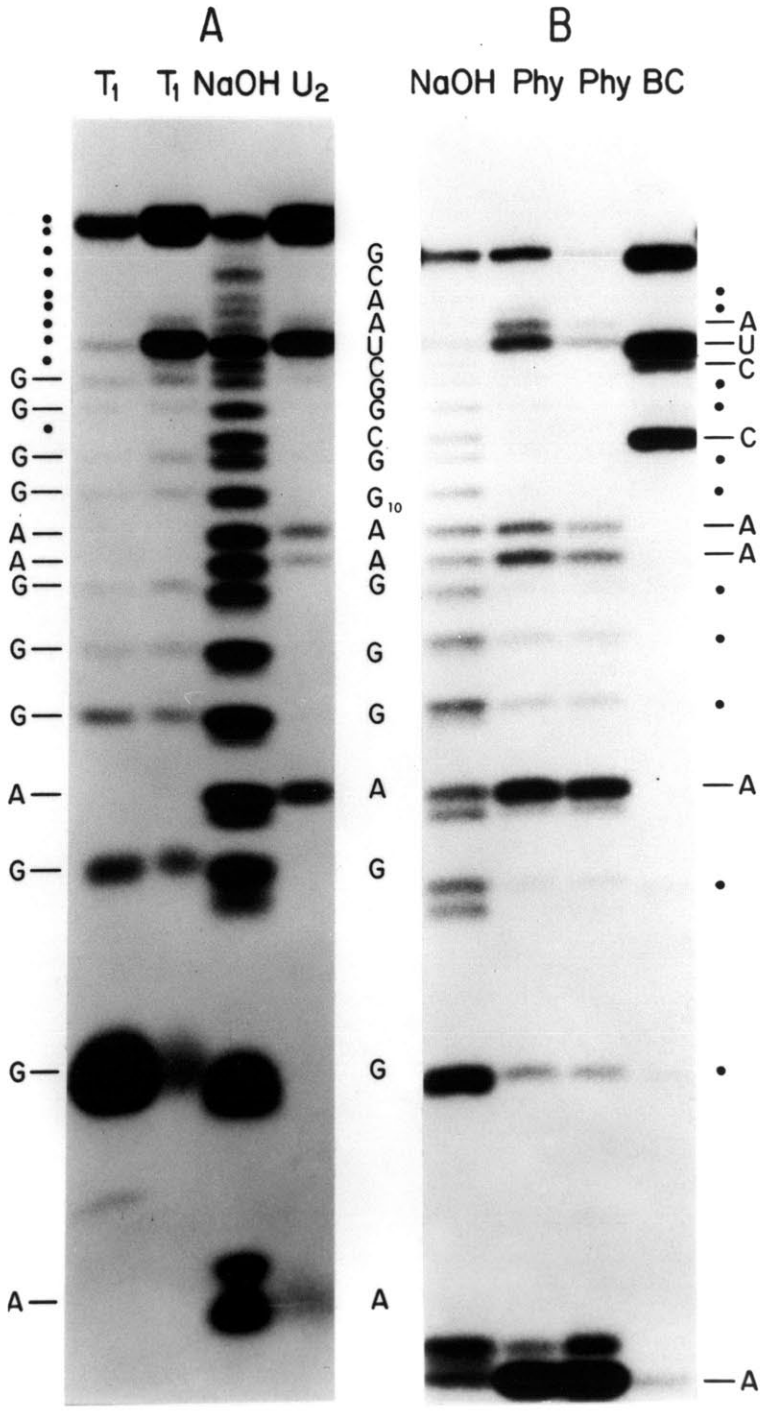
NaOH, partial alkaline digest

U_2, 0.07 units

Phy, 1 µl Physarym ribonuclease I

Phy, 2 µl

BC, 0.04 units B. cereus ribonuclease


A
B
T1
T1
NaOH
U2
NaOH
Phy
Phy
BC
G
G
G
G
A
A
G
G
G
A
G
G
A
G
C
A
A
U
C
G
G
C
G
G10
A
A
G
G
G
A
G
G
A
U
C
C
A
A
A
A

As shown in Figure 8, the mobility shifts that are observed by this method for the long St-1 oligoribonucleotide are consistent with the results of the partial ribonuclease digestions (Figure 7) and establish the following sequence,

5' (p)ppA-G-G-A-G-G-G-A-A-G-G-C-G-G-C-U-A-A-C-G-X-X-X_{OH} 3'.

Comparison of the nucleotide sequences for the long and short St-1 oligoribonucleotides demonstrates that the synthesis of each is initiated at the same site. To determine whether all oligonucleotide synthesis begins at this site, the total St-1 oligoribonucleotide product was digested with ribonuclease T_1 and fingerprinted (data not shown). Greater than 90% of the radioactivity was found in the same 7 digest products that comprised the long St-1 oligoribonucleotide (Figure 3B). It is therefore concluded that all oligonucleotide synthesis catalyzed by the dnaG protein on St-1 single-stranded DNA begins at a unique site and proceeds for 10 to 28 nucleotides before terminating.

Nucleotide sequences for the øK and α3 oligoribonucleotide products

The nucleotide sequences of the oligoribonucleotide products synthesized on øK and α3 single-stranded DNA were determined by the ribonuclease T_1 fingerprinting method as shown in Figures 9 and 10, respectively. The resulting digest patterns appear identical to those obtained for the products synthesized on St-1 DNA (compare with Figure 3). The identity and molar yield for each nucleotide found in these ribonuclease T_1 digests is summarized in Table 7. These results demonstrate that the oligoribonucleotide products synthesized on St-1, øK and α3 DNA have the same nucleotide sequence, and that oligonucleotide synthesis on all three DNA

Figure 8 Partial alkaline digest products of 5' end-labeled long St-1 oligoribonucleotide separated by two-dimensional gel electrophoresis.

5' end-labeled long St-1 oligoribonucleotide was prepared as described in the legend to Figure 7. The product was partially digested in alkali for 50 minutes at 90°C and analysed by two-dimensional gel electrophoresis (described in Methods). Electrophoresis in the first dimension (10% polyacrylamide pH 3.5/7 M urea) was for 24 hours at 200 volts and in the second dimension (20% polyacrylamide pH 8.3/7 M urea) for 32 hours at 300-400 volts. Mobility shifts due to a difference of a G or U residue between oligonucleotide hydrolysis products of successive chain length are nearly vertical and may be distinguished from those due to a difference of an A or C residue, which are more angular.

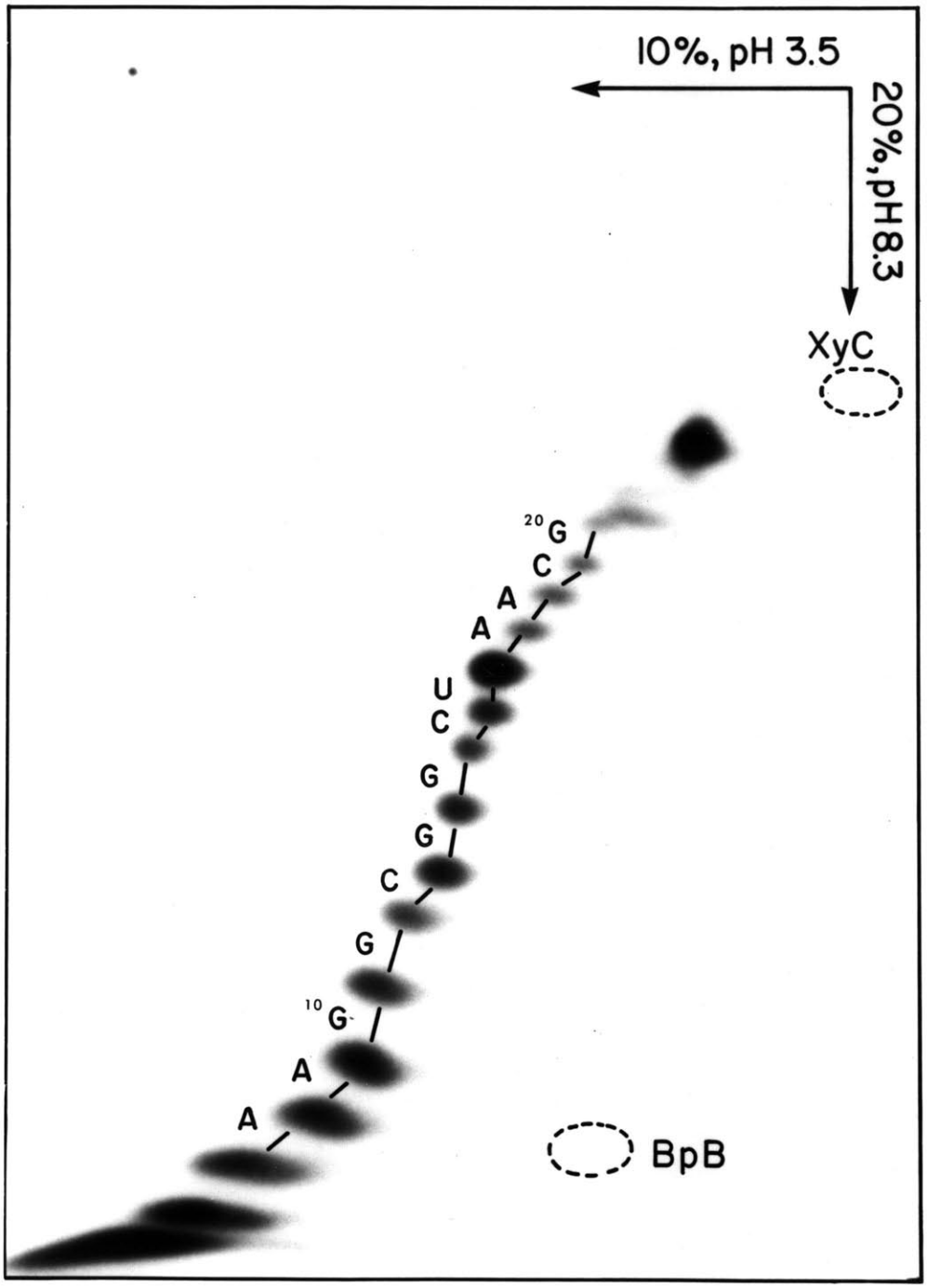
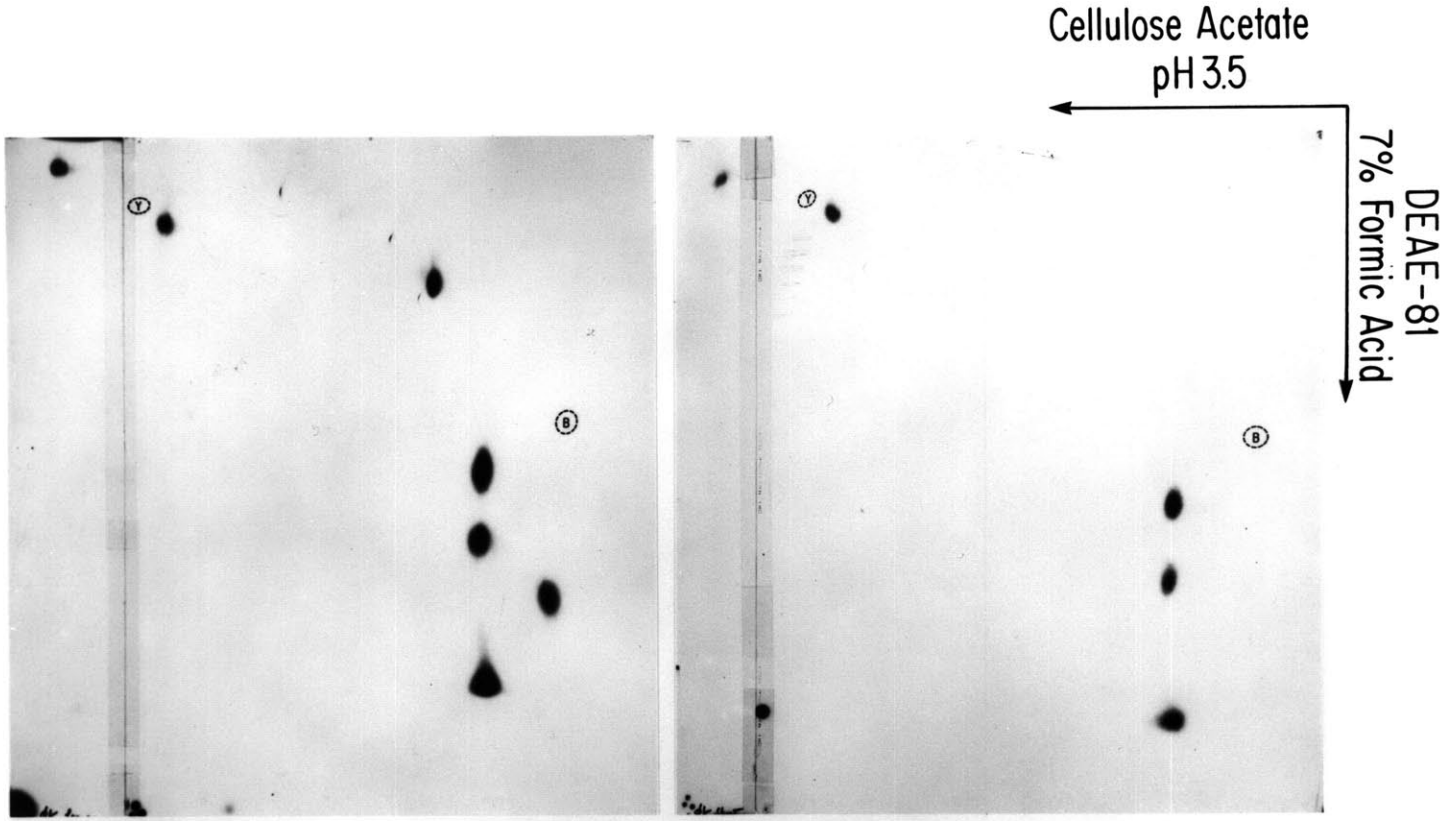

10%, pH 3.5
20%, pH 8.3
XyC
BpB
20 G
C
A
A
U
C
G
G
C
G
10 G
A
A

Figure 9 Ribonuclease T_1 fingerprints of the øK oligoribonucleotide products.

Panel (a) and (b) represent ribonuclease T_1 fingerprints of the long and short oligoribonucleotide products (uniformly radioactive), respectively, synthesized by the dnaG protein on øK single-stranded DNA. The molar yields for each digest product are summarized in Table 7.

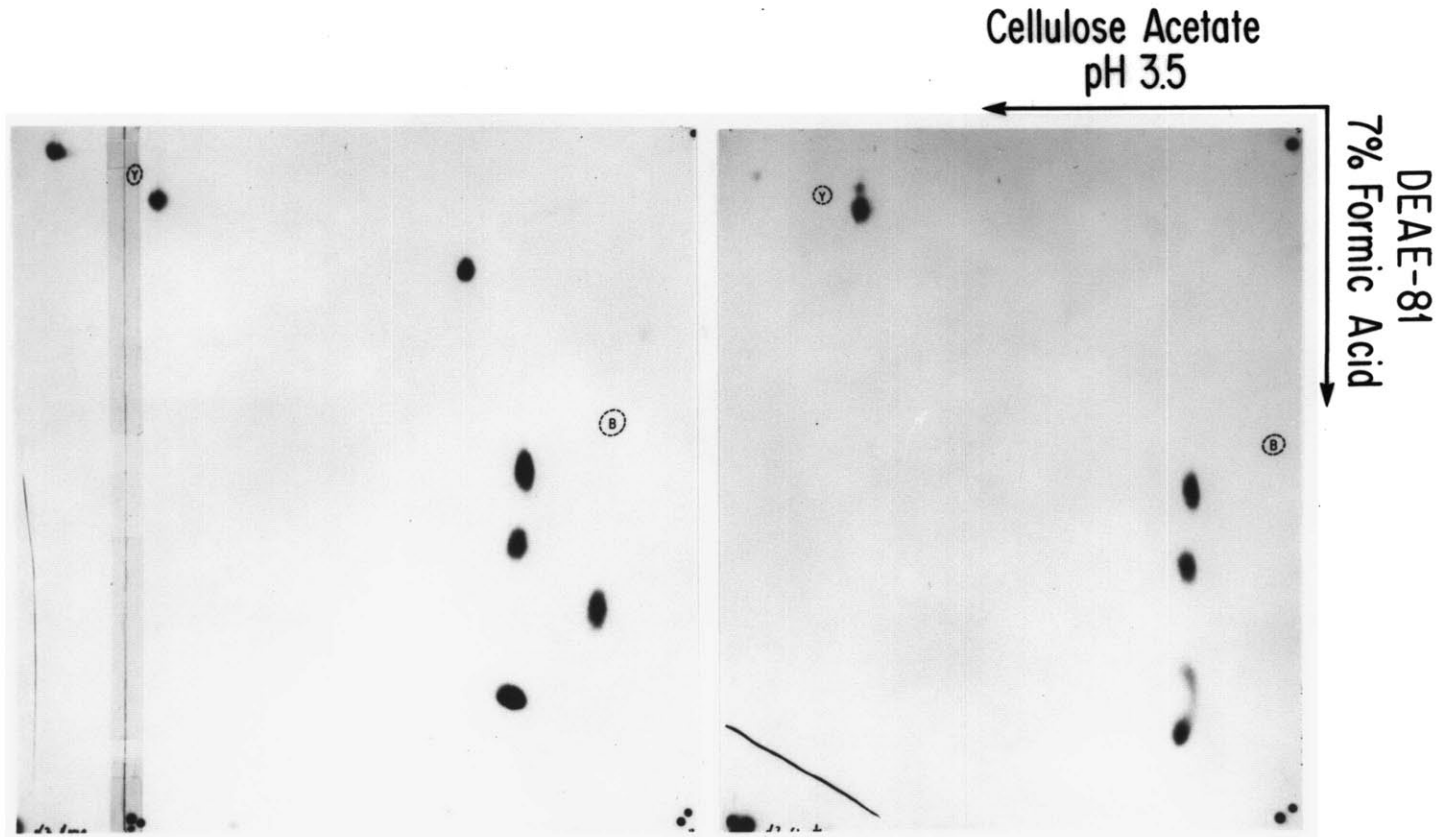


a) ϕK long

b) ϕK short

Figure 10 Ribonuclease T_1 fingerprints of the α3 oligoribonucleotide products.

Panels (a) and (b) represent ribonuclease T_1 fingerprints of the long and short oligoribonucleotide products (uniformly radioactive), respectively, synthesized by the dnaG protein on α3 single-stranded DNA. The molar yields for each digest product are summarized in Table 7.


Cellulose Acetate
pH 3.5
DEAE-81
7% Formic Acid
Y
B
Y
B
a) α3 long
b) α3 short

templates is initiated at a unique site with ATP.

The sequence of the St-1, øK and α3 oligoribonucleotide products is compared with the sequence determined for the product synthesized on G4 DNA (Bouche, Rowen and Kornberg, 1978, D. Capon and S. Wickner, unpublished observations) in Figure 11. These sequences are very similar, differing by only 5 nucleotides out of the first 26. Each sequence may be arranged into a hairpin structure consisting of 8 contiguous base-pairs with a 5 base loop, that is situated 6-7 nucleotides from the start site (Figure 11). The functional significance of this structure is suggested by the two base differences between the St-1 (øK, α3) and G4 products that occur in the hairpin, which compensate to preserve base-pairing (position 9, A → C; position 23, U → G).

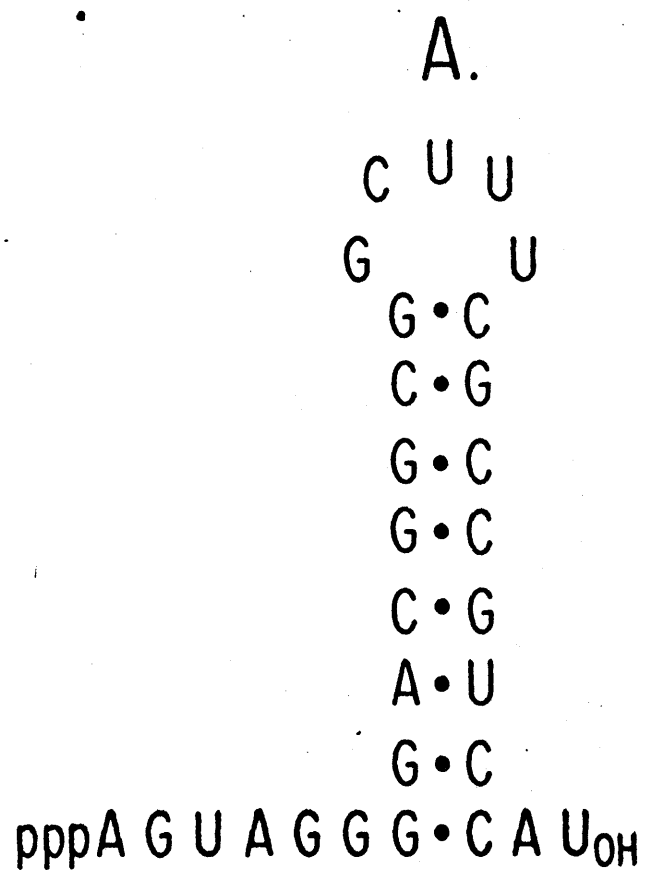
The proposed secondary structure is verified by a partial ribonuclease T_1 digestion of the long St-1 oligoribonucleotide (Figure 7). At an enzyme concentration which leaves at least 1/3 of the oligonucleotide undigested, there is a significant preference for cleavage at single-stranded positions (2, 3 and 5) as compared to hairpin positions (6, 7, 10, 11, 13, 14 and 20). This preference was even more evident when the digestion was performed at a lower temperature (55°C vs. 65°C), which is expected to stabilize base-pairing (data not shown).

Identification of the site at which DNA synthesis is initiated by the dnaG protein on St-1, øK and α3 single-stranded DNA

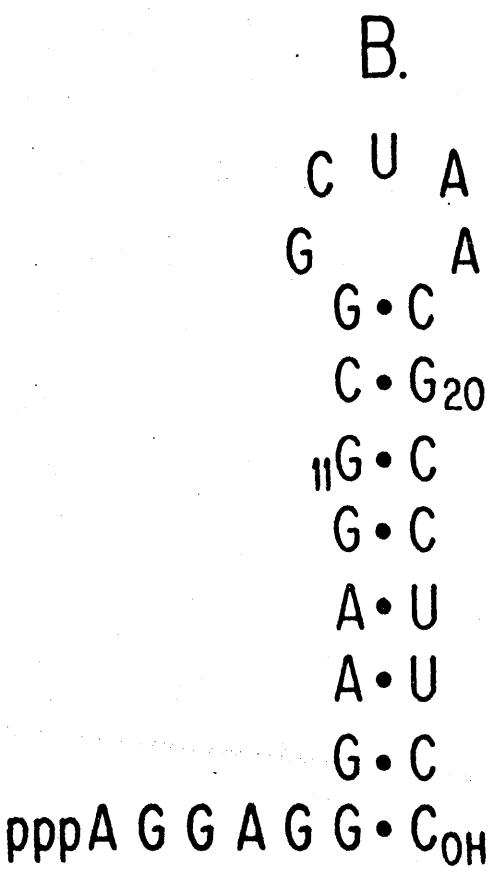
The results presented above suggest that priming of DNA synthesis by the dnaG protein, which is specific for St-1, øK, α3 and G4 DNA but not øx174 DNA in the presence of DNA binding protein (Table 6), involves a

Figure 11 Comparison of nucleotide sequence and secondary structure for the oligonucleotide primers synthesized on St-1, øK, α3 and G4 DNA.

Potential secondary structure is depicted for the oligoribonucleotide products synthesized by the dnaG protein on St-1, øK and α3 single-stranded DNA (panel a) and G4 DNA (panel b, Bouche, Rowen and Kornberg, 1978, D. Capon and S. Wickner, unpublished observations). The short and long St-1 oligoribonucleotides correspond to the first 11 and 23-26 nucleotides, respectively. The sequence for the St-1, øK and α3 products at positions 21-26 was determined by comparing the complemetary sequence found for the origin region of each template (Sims, Capon and Dressler, 1979) as shown in Figure 13.



G4



ST-1 (ϕK, α3)

highly conserved recognition element on these templates. Previous studies had shown that the conversion of G4 single-stranded DNA to RF is initiated *in vivo* at a unique site which is complementary to the oligoribonucleotide primer synthesized by the *dnaG* protein on G4 DNA *in vitro* (Hourcade and Dressler, 1978, Sims and Dressler, 1978). To confirm that primer synthesis on St-, øK and α3 single-stranded DNA is also template-directed, and to determine whether the regions flanking the transcribed sequence are also conserved and therefore possibly contribute to *dnaG* protein recognition, the location at which DNA synthesis is initiated *in vitro* on these phage DNA's was identified (Sims, Capon and Dressler, 1979).

The following procedure was used to locate the replication origins. DNA synthesis on St-1, øK or α3 single-stranded DNA was primed by the *dnaG* protein and DNA binding protein and elongated by DNA polymerase I (labeled in the newly synthesized strand) in the presence of the chain-terminating nucleotide ddTTP to limit the average length of the nascent DNA product. Partially replicated products, prepared at different ddTTP concentrations in this fashion, were digested with restriction endonuclease *Hae* III, and the digest products were resolved by electrophoresis on a denaturing polyacrlamide gel and compared to a marker digest of fully duplex radioactive St-1 RF. If DNA synthesis has been initiated at many sites along the template, then radioactive restriction fragments corresponding to all parts of the phage DNA molecule are expected to be present in the *Hae* III digest of the DNA synthesis products. Alternatively, if DNA synthesis has been initiated at a unique location, then restriction fragments closest to the origin will be preferentially represented in the *Hae* III digest since DNA synthesis will have been terminated soon after starting by the incorporation

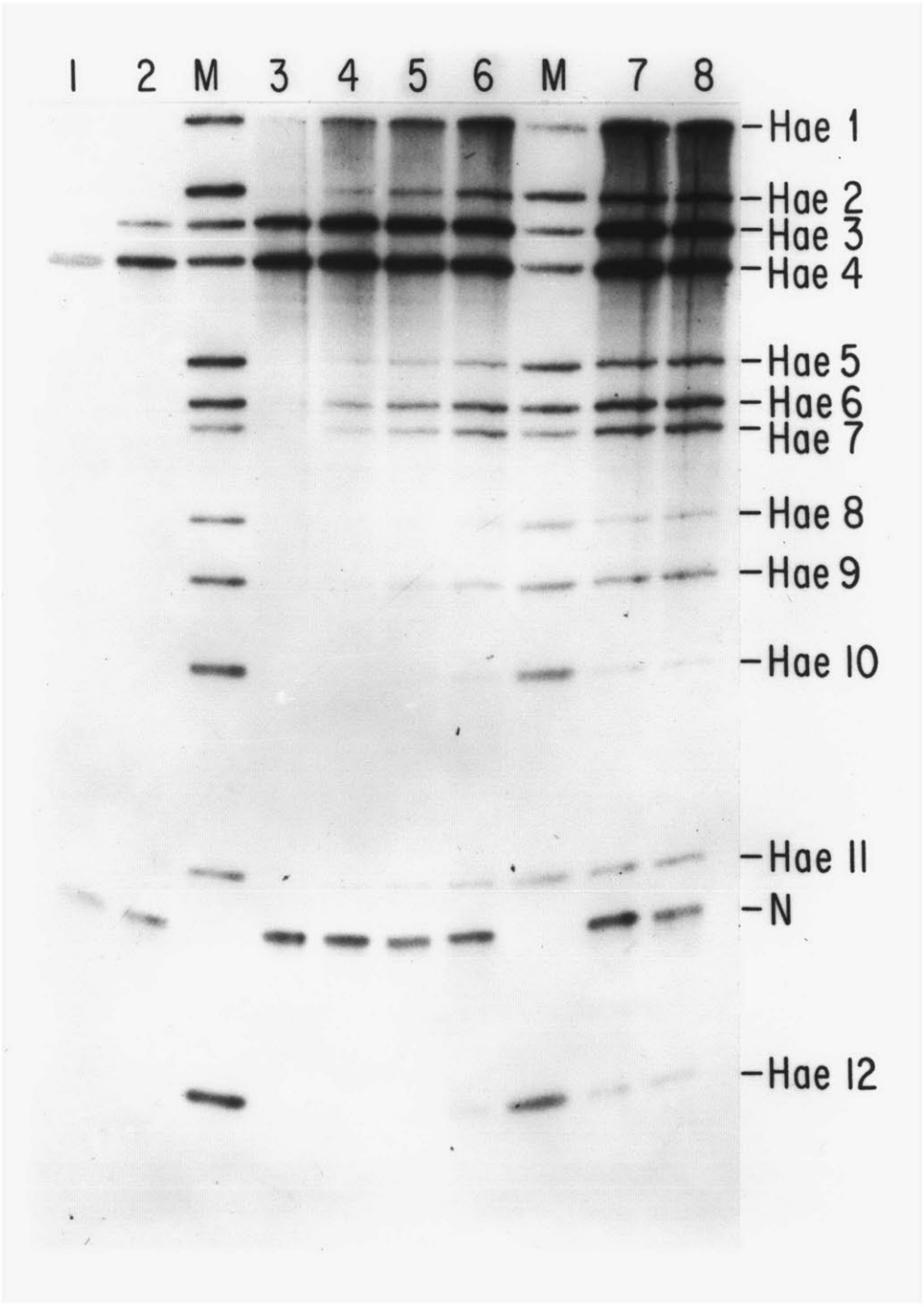
of a ddTMP residue and therefore sufficient to convert only these fragments to double-stranded DNA. This procedure is illustrated for St-1 DNA synthesis in Figure 12.

When St-1 DNA synthesis is performed at a high ddTTP concentration, only three fragments appear in the Hae III digest of the DNA product (lanes 1 and 2). This result is consistent with the interpretation that DNA synthesis is initiated at a unique site on St-1 single-stranded DNA by the dnaG protein. Two of the fragments are identified as Hae 3 and Hae 4 (the third and fourth largest fragments present in a Hae III digest of St-1 RF DNA) by comparison with the marker lane (lane M). The appearance of the third fragment (N), which is 105 nucleotides in length, is consistent with that expected for the portion of the nascent DNA product that extends from the origin to the first Hae III restriction site on St-1 RF DNA. Since Hae 4 is represented to a greater extent than Hae 3 in the digest it is probably the first complete Hae III restriction fragment to lie downstream (by 105 nucleotides) from the origin.

As the concentration of ddTTP in the reaction is lowered, the average length of the nascent DNA product synthesized increases, and fragments corresponding to regions more distal to the origin appear in the Hae III digest (lanes 3 through 7). In the absence of ddTTP, two fragments, Hae 8 and Hae 10, are still poorly represented in the digest and therefore lie furthest downstream from the origin (lane 8). One of these is expected to correspond to the origin-containing fragment, which arises through strand displacement synthesis catalyzed by DNA polymerase I. To confirm the identity of the origin-containing fragment, the same reaction products were analyzed with another restriction endonuclease, Hpa II (data not shown).

Figure 12 Identification of the St-1 replication origin.

Partially duplex St-1 DNA products synthesized in the presence of ddTTP were digested with restriction endonuclease Hae III and electrophoresed on 4 % polyacrylamide gel in the presence of 7 M urea. Reaction mixtures (25 µl) contained reaction buffer (described in Table 5), 0.6 nmole (as nucleotide) St-1 DNA, 0.075 units dnaG protein, 0.66 µg DNA binding protein, 40 mM ADP, 40 µM each dATP, dCTP and dGTP, 5 µM (α-^{32}P) TTP and the following amount of ddTTP: (1) 320 µM, (2) 160 µM, (3) 80 µM, (4) 40 µM, (5) 20 µM, (6) 10 µM, (7) 5 µM, (8) no ddTTP. After 20 minutes at 30°C the reactions were stopped by the addition of 0.05 M EDTA, extracted with phenol and filtered through Sephadex G-50 to remove radioactive substrates. Lanes M represent a Hae III digest of fully duplex St-1 RF DNA which had been radioactively labeled with T4 polynucleotide kinase and (γ-^{32}P) ATP as described in Methods. Each sample was denatured in formamide before being applied to the gel.


1
2
M
3
4
5
6
M
7
8
Hae 1
Hae 2
Hae 3
Hae 4
Hae 5
Hae 6
Hae 7
Hae 8
Hae 9
Hae 10
Hae 11
N
Hae 12

These results demonstrate that DNA synthesis begins 90-95 nucleotides upstream from Hpa 12 and probably within the fragment Hpa 8.

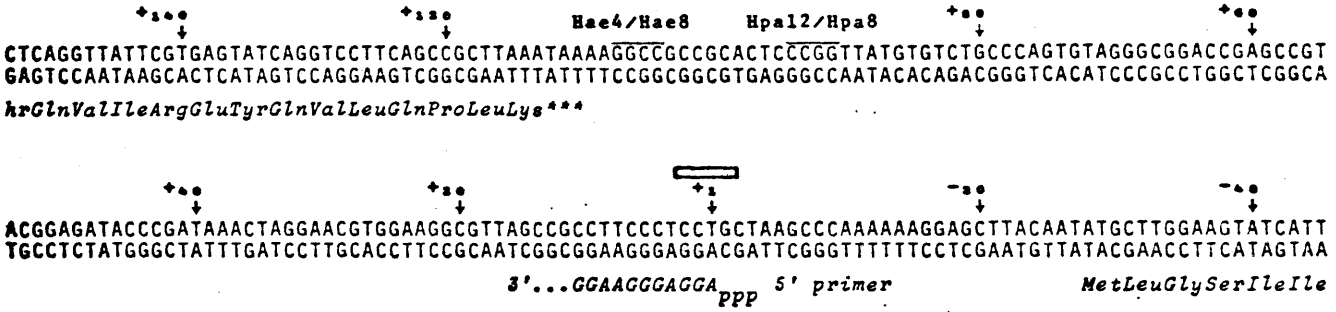
The relationship between the origin-containing and origin-proximal fragments was conclusively established by determining the nucleotide sequence of each (performed by Dr. John Sims). The sequence of the St-1 origin region, as well as those for the øK and α3 origins, which were located by the same method (data not shown), are presented in Figure 13. Examination of these sequences demonstrates that, in each case, a sequence complementary to the oligoribonucleotide primer synthesized by the dnaG protein begins at the location on the DNA determined for the origin of DNA synthesis (indicated by the horizontal bars). Comparison of these sequences, which each lie within an intercistronic region of approximately 135 nucleotides, reveals extensive nucleotide homology on either side of the origin, which does not extend into the adjacent coding regions. A complete discussion of sequence homology, secondary structure and protein coding capacity, which is beyond the scope of this thesis, has been presented (Sims, Capon and Dressler, 1979).

Conditions which affect the length of primer synthesis on St-1 DNA

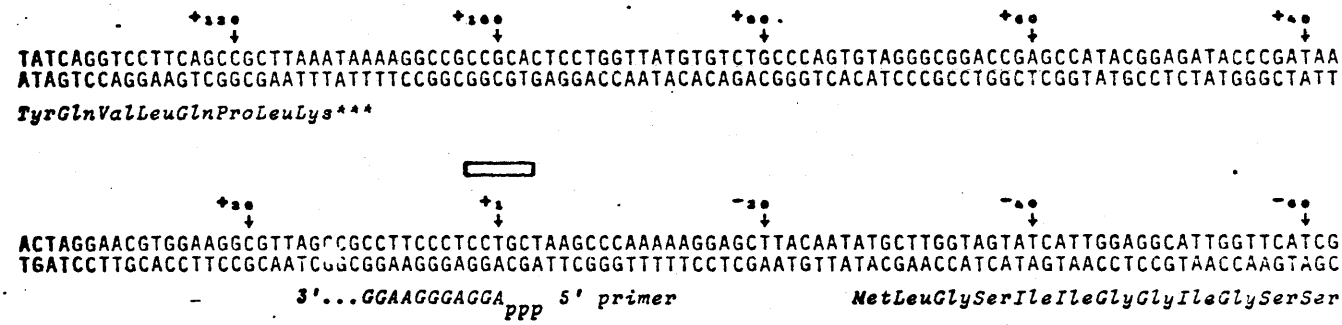
As demonstrated earlier, oligoribonucleotide synthesis catalyzed by the dnaG protein on St-1, øK and α3 single-stranded DNA may proceed for a distance of 10 to 28 nucleotides, but is terminated most frequently after 11 or 22-26 nucleotide additions (Figure 2). Since primer synthesis is initiated at a unique site on these templates, the length of the products corresponds to specific sequences on the DNA. Accordingly, the mechanism of primer elongation and completion was studied by examining

Figure 13 Nucleotide sequences of the St-1, øK and α3 negative strand initiation sites.

Panels (a), (b) and (c) represent the nucleotide sequences surrounding the site at which DNA synthesis is initiated on St-1, øK and α3 single-stranded (determined by Dr. John Sims), respectively, which was located by the *in vitro* mapping procedure described in Figure 12 and the text (taken from Figures 4, 5 and 6 of Sims, Capon and Dressler, 1979, with the authors' permission). The upper strand corresponds to the viral single-stranded DNA. Each horizontal bar indicates the site identified as the negative strand origin. Within this region begins a sequence complementary to the oligoribonucleotide primer synthesized by the *dnaG* protein (at the position marked +1; compare with Figure 11).


Hae4/Hae8
Hpa12/Hpa8
CTCAGGTTATTCGTGAGTATCAGGTCCTTCAGCCGCTTAAATAAAAGGCCGCCGCACTCCCGGTTATGTGTCTGCCCAGTGTAGGGCGGACCGAGCCGT
GAGTCCAATAAGCACTCATAGTCCAGGAAGTCGGCGAATTTATTTTCCGGCGGCGTGAGGGCCAATACACAGACGGGTCACATCCCGCCTGGCTCGGCA
hrGlnValIleArgGluTyrGlnValLeuGlnProLeuLys***
ACGGAGATACCCGATAAACTAGGAACGTGGAAGGCGTTAGCCGCCTTCCCTCCTGCTAAGCCCAAAAAAGGAGCTTACAATATGCTTGGAAGTATCATT
TGCCTCTATGGGCTATTTGATCCTTGCACCTTCCGCAATCGGCGGAAGGGAGGACGATTCGGGTTTTTTCCTCGAATGTTATACGAACCTTCATAGTAA
3'...GGAAGGGAGGA ppp 5' primer
MetLeuGlySerIleIle

a) St-1


TATCAGGTCCTTCAGCCGCTTAAATAAAAGGCCGCCGCACTCCTGGTTATGTGTCTGCCCAGTGTAGGGCGGACCGAGCCATACGGAGATACCCGATAA
ATAGTCCAGGAAGTCGGCGAATTTATTTTCCGGCGGCGTGAGGACCAATACACAGACGGGTCACATCCCGCCTGGCTCGGTATGCCTCTATGGGCTATT
TyrGlnValLeuGlnProLeuLys***
ACTAGGAACGTGGAAGGCGTTAGCCGCCTTCCCTCCTGCTAAGCCCAAAAAGGAGCTTACAATATGCTTGGTAGTATCATTGGAGGCATTGGTTCATCG
TGATCCTTGCACCTTCCGCAATCGGCGGAAGGGAGGACGATTCGGGTTTTTCCTCGAATGTTATACGAACCATCATAGTAACCTCCGTAACCAAGTAGC
3'...GGAAGGGAGGA ppp 5' primer
MetLeuGlySerIleIleGlyGlyIleGlySerSer

b) ϕK


CAGGTTATTCATGAATACCAAGTCCTTCAGCCGCTTAAATAAAAGGCTGCCGCACTCCCGGTTAGATGCCTGCCCAGTGTAGGGCAGACCGAGCCGTAC
GTCCAATAAGTACTTATGGTTCAGGAAGTCGGCGAATTTATTTTCCGACGGCGTGAGGGCCAATCTACGGACGGGTCACATCCCGTCTGGCTCGGCATG
GlnValIleHisGluTyrGlnValLeuGlnProLeuLys***
GGAGATACCCGATAAACTAGGAACGTGGAAGGCGTTAGCCGCCTTCCCTCCTGCTAAGCCCAAAAAGGAGTTACAGCGATGTTAGGTGCCGTTGTTGGT
CCTCTATGGGCTATTTGATCCTTGCACCTTCCGCAATCGGCGGAAGGGAGGACGATTCGGGTTTTTCCTCAATGTCGCTACAATCCACGGCAACAACCA
3'...GGAAGGGAGGA ppp 5' primer
MetLeuGlyAlaValValGly

c) α3

the factors which influence the length of the product synthesized in the absence of DNA synthesis.

An investigation of the rate-limiting steps in primer formation is shown in Figure 14. Lanes (1) through (5) represent primer synthesis on St-1 DNA in the presence of 25 μM rNTP's after 0.5, 1, 2, 5 and 10 minutes of incubation at 30°C.[1] Under these conditions, the rate of primer synthesis is linear for 2 minutes (Figure 15). Despite the steady accumulation of product 10-12 nucleotides long during this period, there is a complete absence of product smaller than 10 nucleotides even after 0.5 minutes.[2] This demonstrates that the overall rate of primer formation is limited by primer chain initiation rather than elongation.

Primer initiation is not the most rate-limiting step in the formation of primers longer than 10-12 nucleotides, however. It is evident that products 22-26 nucleotides long are initially synthesized at a 5-10 fold slower rate than products 10-12 nucleotides in length (Figure 14). This suggests that a barrier to primer elongation is situated near position 11. Again, the absence of products of intermediate lengths is readily apparent. This may indicate that during elongation the dnaG protein does not dissociate from the template between successive nucleotide additions unless confronted with a termination site.

[1] The same amount of radioactivity was electrophoresed in each lane.

[2] To confirm this, reaction mixtures were analyzed directly by electrophoresis at pH 1.9 on DEAE-81 paper after enzymatic dephosphorylation which permits quantitative recovery of products as small as a dinucleotide (see Figure 6).

Figure 14 Kinetic analysis of oligoribonucleotide primer synthesis on St-1 DNA

Reaction mixture (25 µl) contained 3.0 nmoles of St-1 DNA, 0.7 units of dnaG protein, 3.3 µg of DNA binding protein and 25 µM each ATP, CTP, UTP and (α-^{32}P)GTP (78 Ci/mmole). After 0, 0.5, 1, 2, 5 and 10 minutes at 30°C, a 4 µl aliquot was withdrawn, stopped by the addition of 0.05 M EDTA and filtered through Sepharose 6B to remove unreacted substrate as described in Methods. A portion of each product containing the same amount of radioactivity was electrophoresed on a 20% polyacylamide/7 M urea gel as shown. The time course of (α-^{32}P)GMP incorporation is shown in Figure 15.

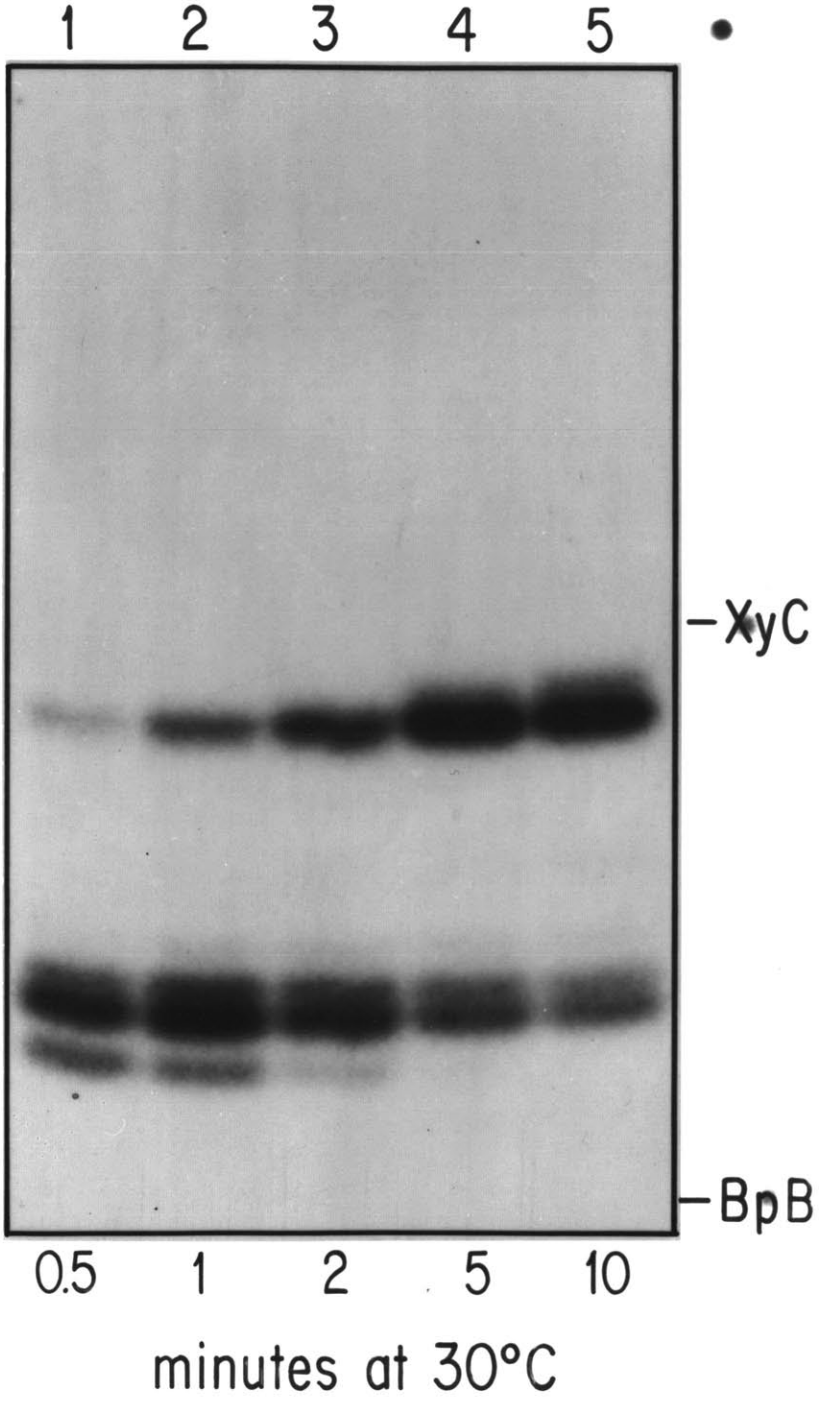
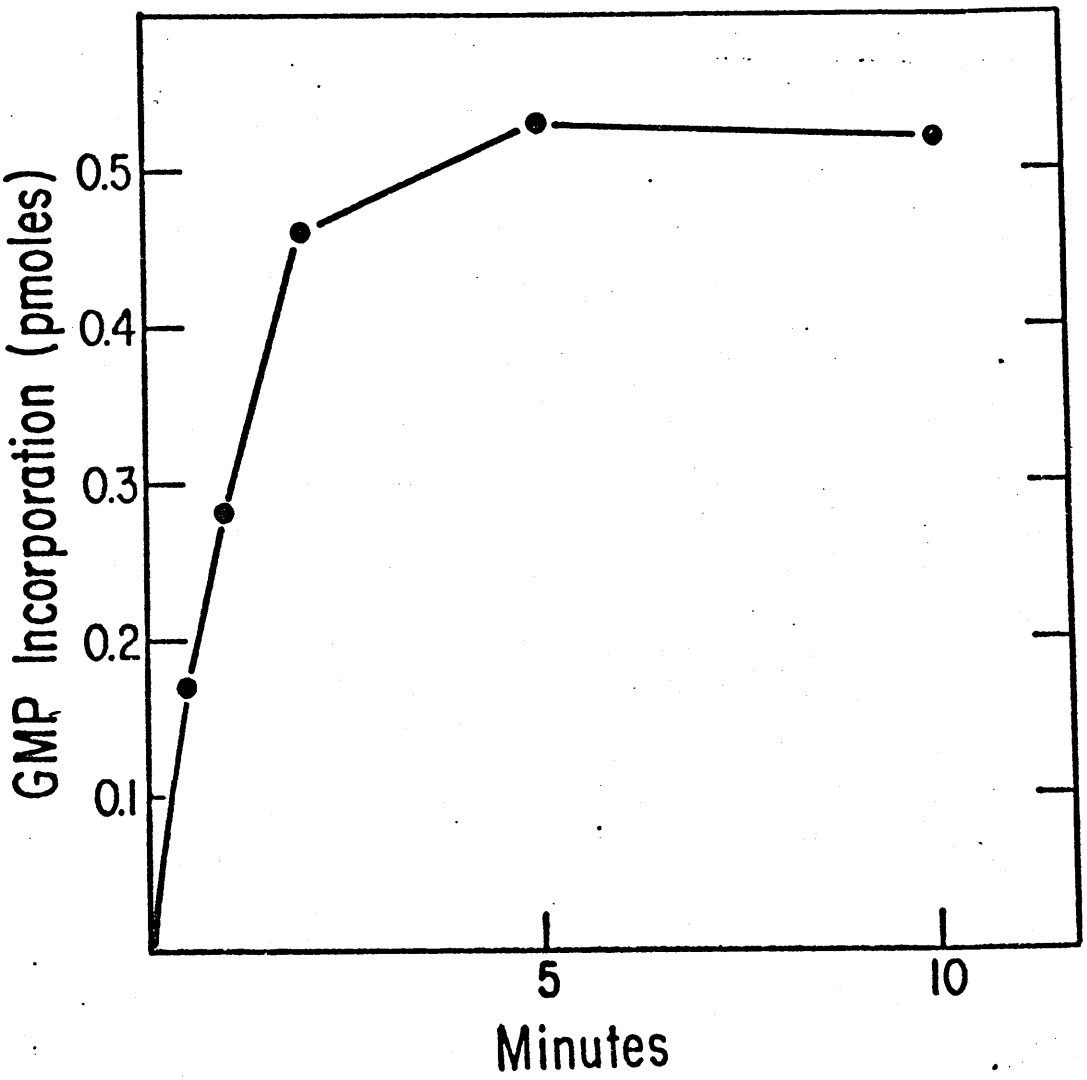

1
2
3
4
5
XyC
BpB
0.5
1
2
5
10
minutes at 30°C

Figure 15 Time course of oligoribonucleotide synthesis on St-1 DNA

Reaction mixtures are described in Figure 14.


GMP Incorporation (pmoles)
0.5
0.4
0.3
0.2
0.1
5
10
Minutes

One question raised by these results concerns why primer synthesis terminates at positions 10-12 when it may otherwise extend the entire distance of the hairpin region. The location of this site relative to base-pairing within the hairpin suggests that secondary structure alone does not comprise the barrier to subsequent primer elongation (Figure 11). Additional factors may also contribute, such as interactions with other regions of the template or with DNA binding protein as the opposite strand of the hairpin becomes displaced during synthesis. The likelihood that the dnaG protein will proceed past a barrier of such a nature may nevertheless depend upon conditions that affect the stability of the hairpin.

To test this hypothesis, parameters that affect secondary structure were examined for their ability to influence the length of primer synthesis. The effect of raising the salt concentration, which is expected to stabilize base-pairing and therefore increase termination, is shown in Figure 16. Panels (A) and (B) represent primer synthesis on St-1 DNA in the presence of 0.15 M KCl and 0.35 M KCl after 0.5, 1.5, 5 and 15 minutes of incubation at 30°C.[1] In agreement with the prediction made, there is no elongation of the product beyond positions 10-12 at the higher salt concentration. In addition, the initial rate of primer synthesis is two-fold less at 0.35 M KCl than at 0.15 M KCl (Figure 17). Since most of the product formed after 0.5 minutes is 10-12 nucleotides in each case, this suggests that the rate of primer initiation is also reduced at the higher salt concentration.

The effect of varying the reaction temperature upon primer length was similarly investigated as shown in Figure 18. Panels (A) and (B) represent primer synthesis on St-1 DNA at 23°C or 37°C after 0.5, 1.5,

Figure 16 Effect of salt concentration on the length of the oligoribonucleotide primer synthesized on St-1 DNA

Reaction mixtures (25 µl) contained either 0.15 M KCl or 0.35 M KCl, and 3.0 nmoles St-1 DNA, 0.7 units of dnaG protein, 3.3 µg DNA binding protein and 25 µM each ATP, CTP, UTP and (α-^{32}P)GTP (67 Ci/mmole). After 0.5, 1.5, 5 and 15 minutes at 30°C, a 6 µl aliquot was withdrawn, stopped by the addition of 0.05 M EDTA and filtered through Sepharose 6B to remove unreacted substrate. A portion of each reaction product containing the same amount of radioactivity was electrophoresed on a 20% polyacrylamide/7 M urea gel as shown. The time course of (α-^{32})GMP incorporation (per 25 1 reaction volume) is shown in Figure 17.

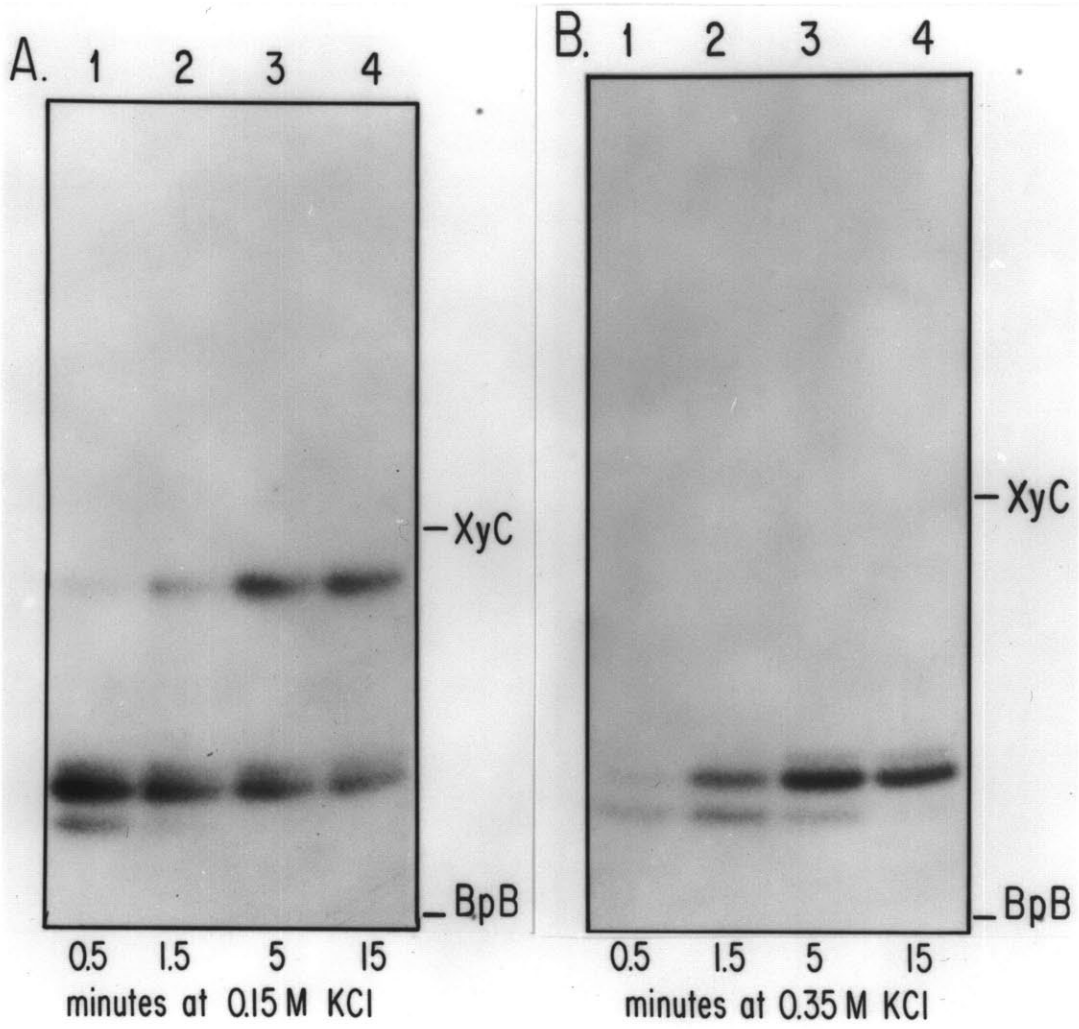

A.
1
2
3
4
XyC
BpB
0.5
1.5
5
15
minutes at 0.15 M KCl
B.
1
2
3
4
XyC
BpB
0.5
1.5
5
15
minutes at 0.35 M KCl

Figure 17 Time course of oligoribonucleotide primer synthesis on St-1 DNA at different salt concentrations

Reaction mixtures are described in Figure 16.

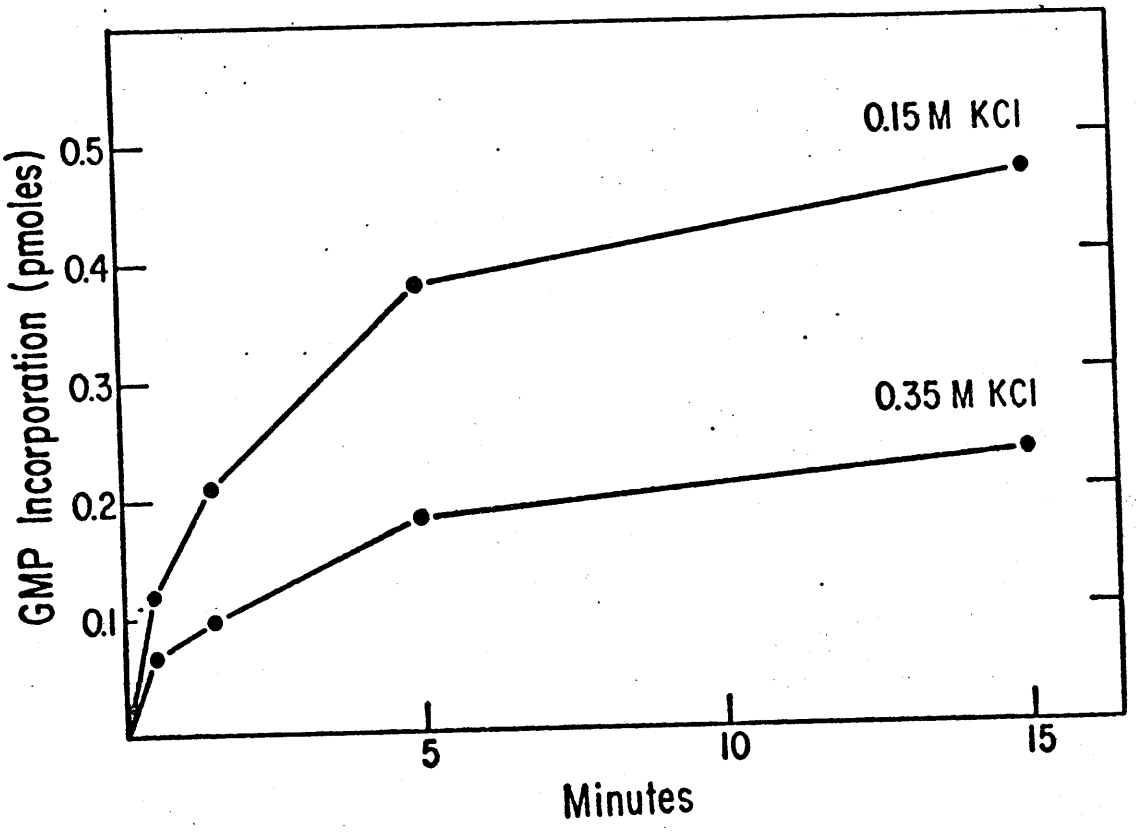
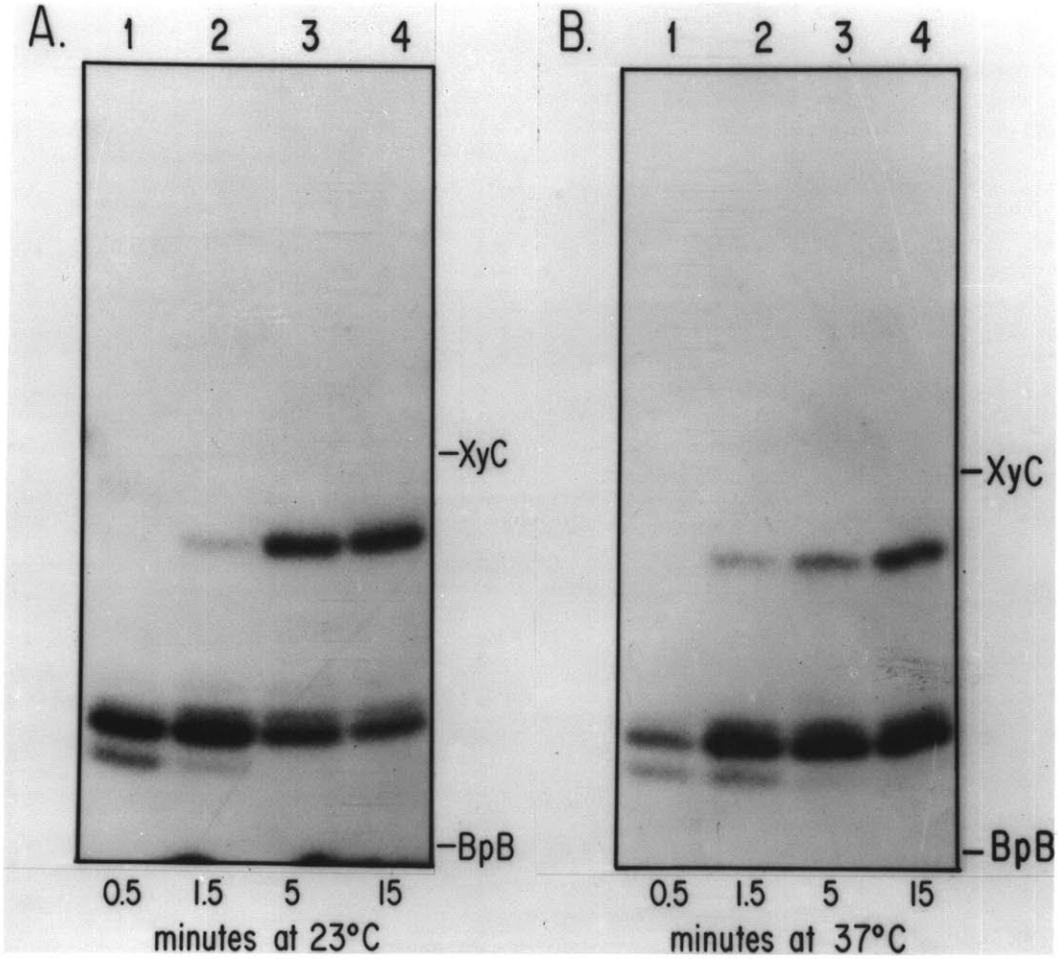

0.15 M KCl
0.35 M KCl
GMP Incorporation (pmoles)
0.5
0.4
0.3
0.2
0.1
5
10
15
Minutes

Figure 18 Effect of temperature on the length of the oligoribonucleotide primer synthesized on St-1 DNA

Reaction mixtures (25 µl) contained 3.0 nmoles of St-1 DNA, 0.7 units of dnaG protein, 3.3 µg of DNA binding protein and 25 µM ATP, CTP, UTP and (α-^{32}P)GTP (58 Ci/mmole). After 0.5, 1.5, 5 and 15 minutes at either 23°C or 37°C, a 6 µl aliquot was withdrawn, stopped by the addition of 0.05 M EDTA and filtered through Sepharose 6B to remove unreacted substrate. A portion of each product containing the same amount of radioactivity was electrophoresed on a 20% polyacrylamide/7 M urea gel as shown. The time course of (α-^{32}P)GMP incorporation (per 25 µl volume) is shown in Figure 19.


A.
1
2
3
4
XyC
BpB
0.5
1.5
5
15
minutes at 23°C
B.
1
2
3
4
XyC
BpB
0.5
1.5
5
15
minutes at 37°C

5 and 15 minutes of incubation.[1] The formation of products longer than 10-12 nucleotides after 0.5 minutes is significantly slower at both 23°C and 37°C than at 30°C (compare with Figure 14). Since the initial rate of primer synthesis at 23°C (Figure 19) is comparable to the rate at 30°C (Figure 15), these results are consistent with an increase in termination at positions 10-12 at the lower temperature. The effect on elongation at 37°C is not as obvious because the rate of primer synthesis is 3-4 fold less at this temperature (Figure 19).

To determine whether elongation beyond positions 10-12 is affected by the rate of primer synthesis, the effect of varying the concentration of substrates was examined as shown in Figure 20. Lanes (1) through (4) represent primer synthesis on St-1 DNA in the presence of 1.5, 5, 15 and 50 μM rNTPs after 20 minutes of incubation at 30°C.[1] The barrier to elongation is progressively overcome as the rNTP concentration is increased. Interestingly, higher rNTP concentrations are required for the synthesis of primers longer than 10-12 nucleotides than for effective primer initiation. The initial rate of primer synthesis, which reflects the rate of primer initiation since this step is rate-limiting (see above), exhibits an apparent K_m of 5 μM for rNTPs (Figure 21). In contrast, 15-25 μM rNTPs is necessary for extensive formation of primers 22-26 nucleotides long (Figure 20).

Lanes (4) and (5) compare the length of the products synthesized in the absence and presence of 50 μM dNTPs in reactions containing 50 μM rNTPs (Figure 20). More product is elongated past the barrier in the presence of dNTPs, suggesting that rNTPs and dNTPs exert a similar effect in making primer synthesis more processive. This was confirmed by the

Figure 19 Time course of oligoribonucleotide primer synthesis on St-1 DNA at different temperatures

Reactions are described in Figure 18.

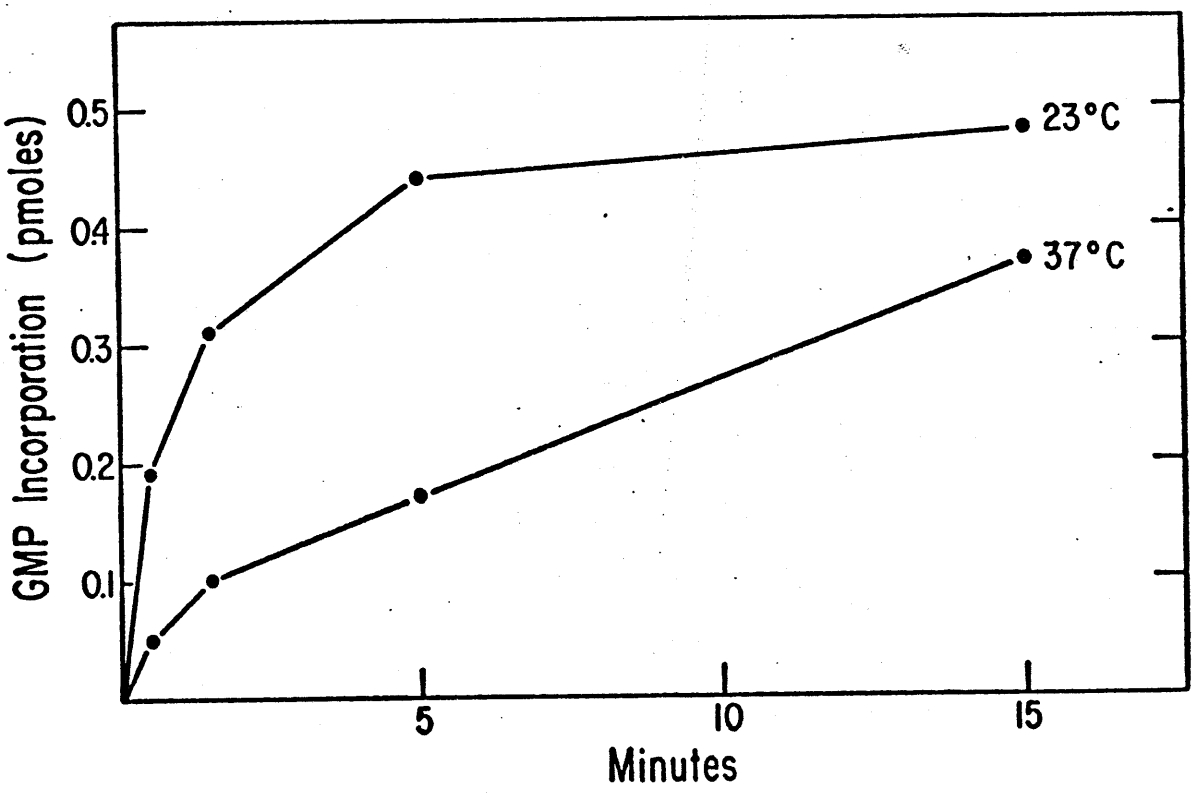

GMP Incorporation (pmoles)
0.5
0.4
0.3
0.2
0.1
23°C
37°C
5
10
15
Minutes

Figure 20 Effect of rNTP concentration on the length of the oligoribonucleotide primers synthesized on St-1 DNA.

Reaction mixtures (25 µl) contained 3 nmoles of St-1 DNA, 0.7 units of dnaG protein, 3.3 µg of DNA binding protein and the indicated concentration of GTP, CTP, UTP and (α-^{32}P) ATP (10-100 Ci/mmole). The reaction shown in lane 5 contained all 4 rNTPs and all 4 dNTPs at 50mM each. After 30 minutes at 30°C, unreacted NTPs were removed from each reaction mixture by Sepharose 6B chromatography, and an aliquot of each product containing the same amount of radioactivity was electrophoresed on a 20% polyacrylamide gel containing 7 M urea as shown. The total (α-^{32}P) AMP incorporation at each rNTP concentration was as follows: (1) 1.5 µM-0.03 pmoles, (2) 5 µM-0.30 pmoles, (3) 15 µM-0.36 pmoles, and (4) 50 µM-0.45 pmoles.

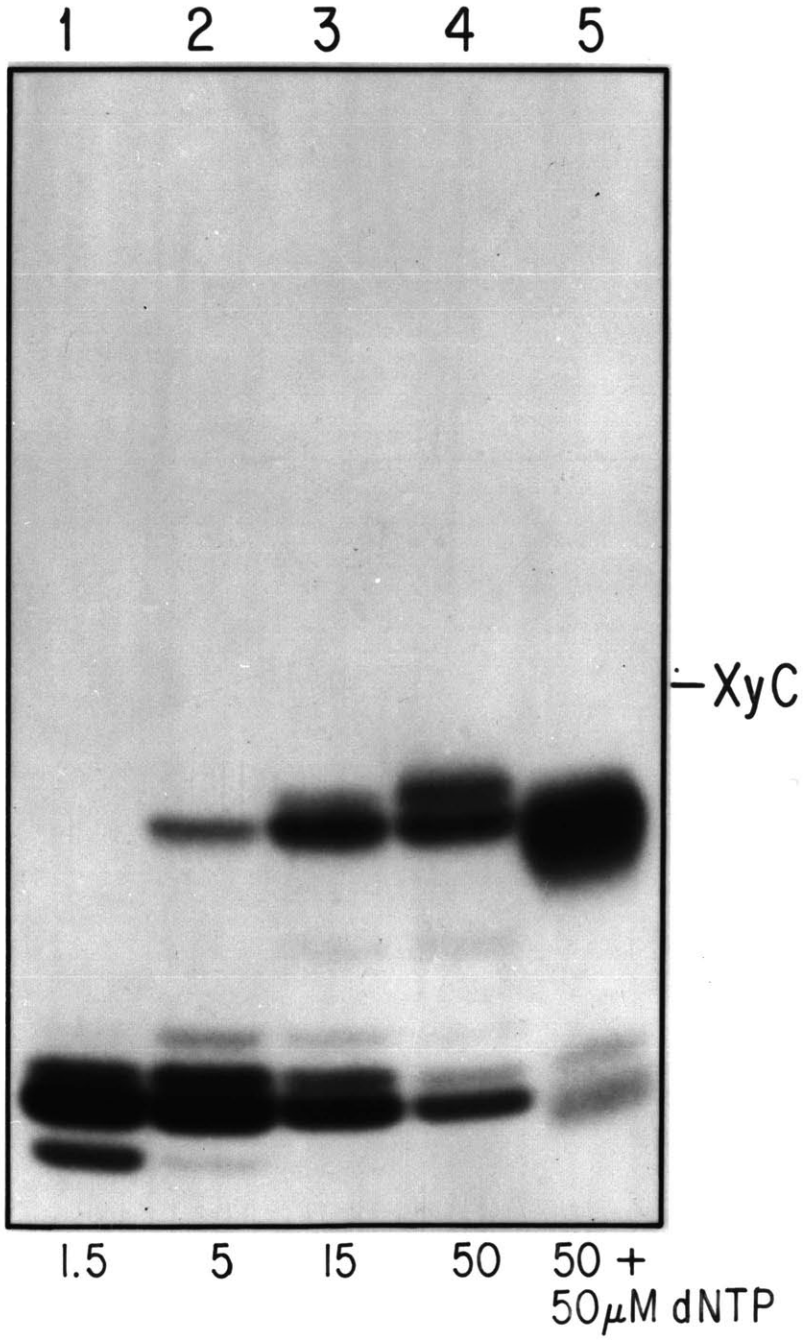
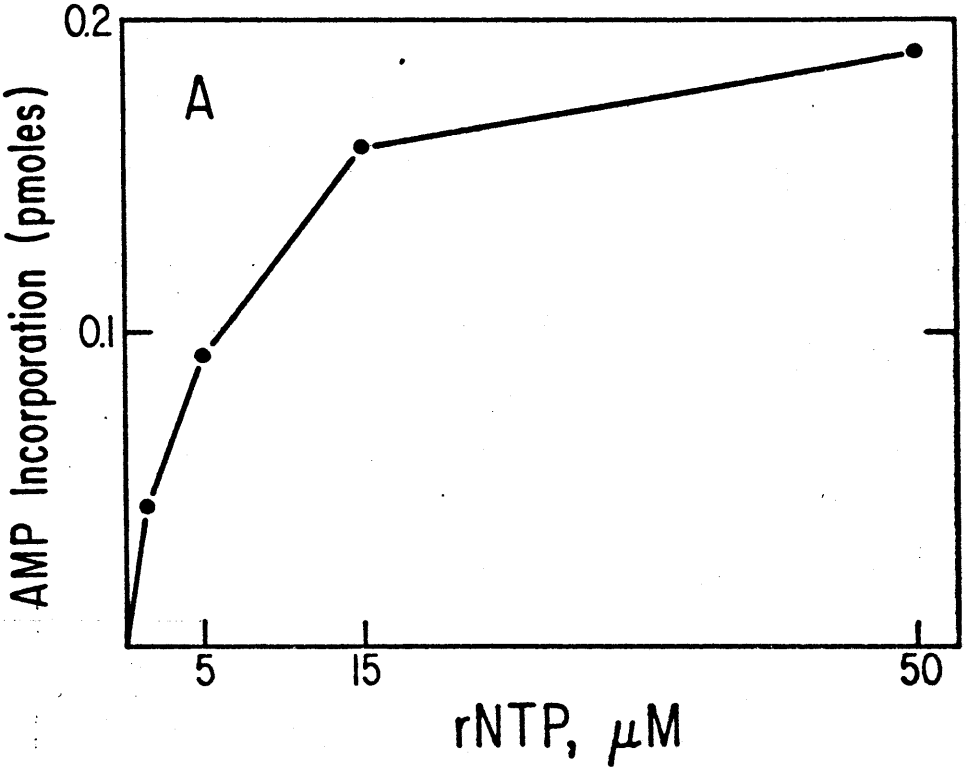
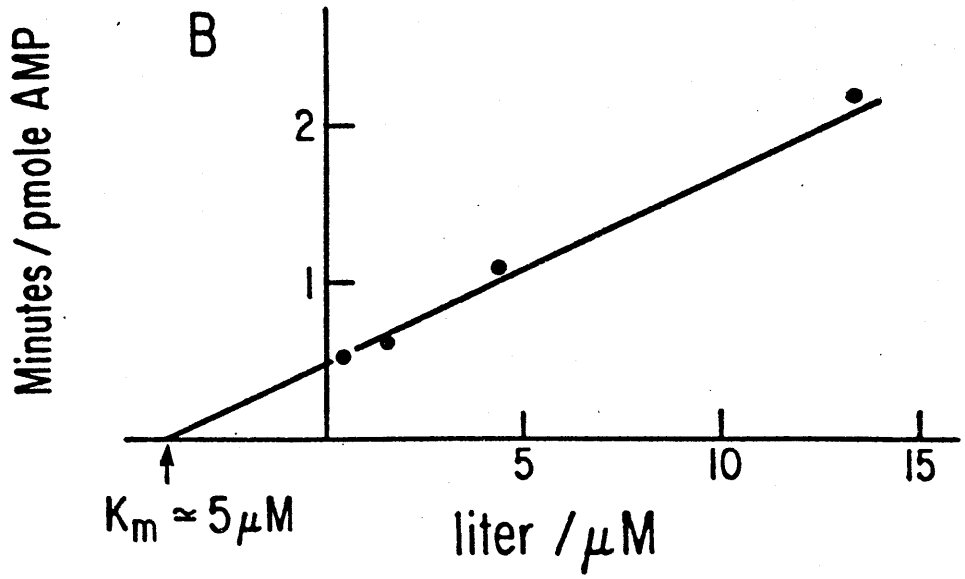

1
2
3
4
5
XyC
1.5
5
15
50
50 +
50μM dNTP
rNTP conc., μM

Figure 21 Effect of rNTP concentration on the rate of oligoribonucleotide primer synthesis on St-1 DNA.

Reaction mixtures (5 µl) contained 0.75 nmoles of St-1 DNA, 0.7 µg of DNA binding protein, 0.12 units of dnaG protein, and 1.5, 5, 15 or 50 µM each of GTP, CTP, UTP and (α-^{32}P)ATP (160 Ci/mmole). After 1 minute at 30°C, each reaction was stopped by the addition of 0.05 M EDTA, filtered through Sepharose 6B to remove unreacted substrate, and AMP incorporation was determined (A). A double-reciprocal plot (Lineweaver-Burke) of the rate of AMP incorporation vs. rNTP concentration indicates an apparent K_m of 5 µM for rNTPs (B).


A
0.2
0.1
AMP Incorporation (pmoles)
5
15
50
rNTP, μM


B
2
1
Minutes / pmole AMP
5
10
15
$K_m \approx 5\mu M$
liter / μM

experiment shown in Figure 22. Lanes (1) through (5) represent primer synthesis on St-1 DNA in the presence of 5 μM rNTPs and 0, 1.5, 5, 15 or 50 μM dNTPs after 20 minutes of incubation at 30°C.[1] As the concentration of dNTPs is increased, the barrier at positions 10-12 becomes less rate-limiting to primer elongation. In addition, there is a corresponding decrease in the extent of rNMP incorporation (Fig. 22), suggesting that rNTPs and dNTPs compete as substrates for primer synthesis.

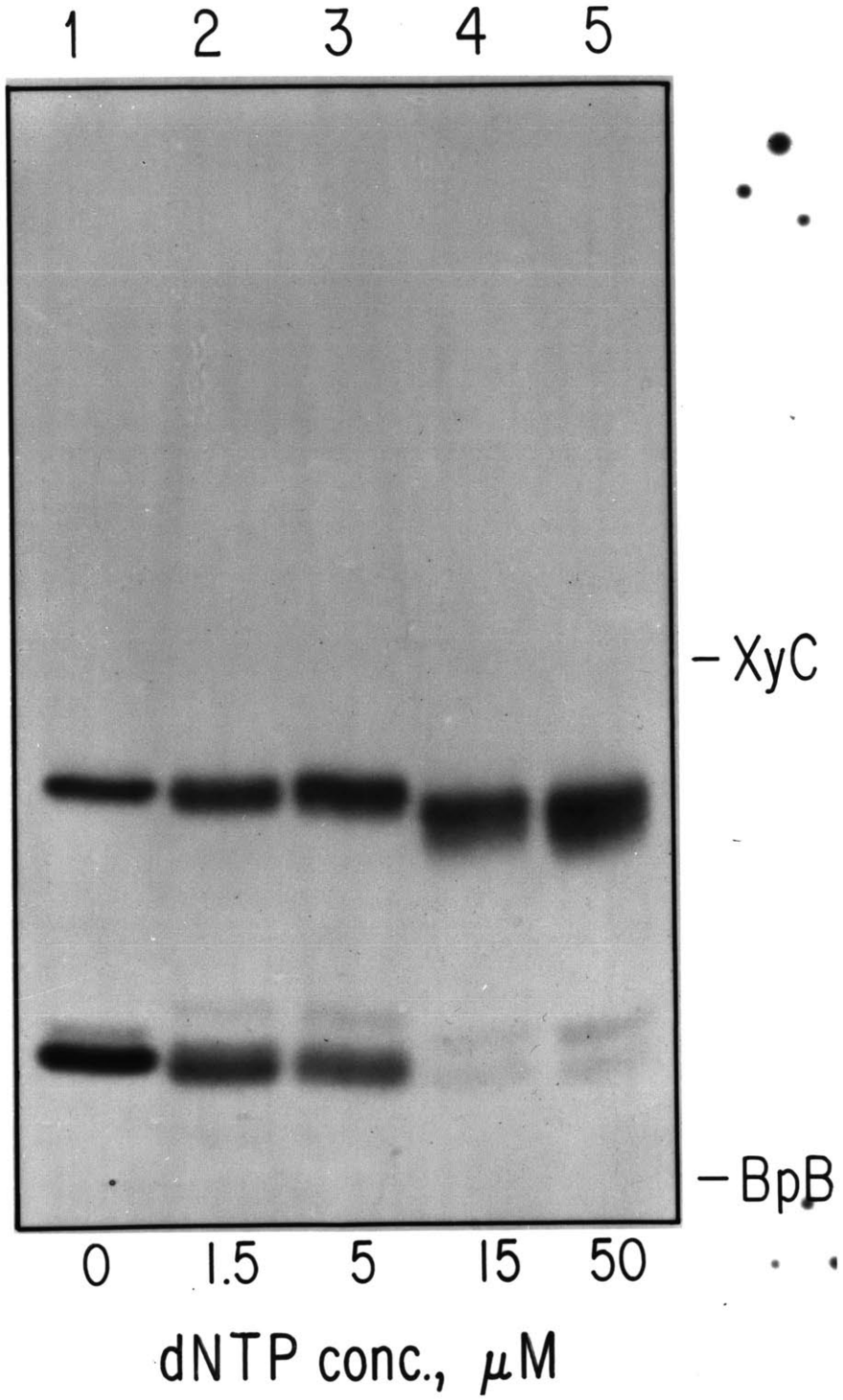
The relative contribution of rNTPs and dNTPs to primer synthesis on St-1 single-stranded DNA

Since the ability of the dnaG protein to utilize both ribonucleotide and deoxyribonucleotide substrates presumably reflects an essential aspect of the enzyme's physiological function, it was of interest to clarify the role of these substrates in primer formation. The relative efficiency with which rNTPs and dNTPs are incorporated at specific positions in the primer synthesized on St-1 DNA has been investigated. The experimental approach consists in sequencing the collection of hybrid primers made in the presence of ATP, GTP and either dATP or dGTP. Under these conditions, primer synthesis terminates at position 11 since CTP or dCTP are required for further elongation to occur, resulting in hybrids of the sequence $pppAGGAGGGAAGG_{OH}$ (Figure 11). As demonstrated below, dATP and dGTP are incorporated at every position that is occupied by the homologous rNTP.

The positional preference for the utilization of ATP and dATP was determined as follows. Products were labeled with (α-^{32}P)GTP during synthesis in order to distinguish between the effect of dATP on total primer synthesis and on the incorporation of ATP. Hybrid primers were

Figure 22 Effect of dNTP concentration on the length of the primer synthesized on St-1 DNA.

Reaction mixtures (10 µl) contained 1.5 nmoles of St-1 DNA, 1.4 µg of DNA binding protein, 0.45 units of dnaG protein, 5 µM each of GTP, CTP, UTP and (α-^{32}P)ATP (260 Ci/mmole), and the indicated concentration of each of the four dNTPs. After 20 minutes at 30°C, each mixture was filtered through Sepharose 6B to remove unreacted substrate, and an aliquot of each product containing the same amount of radioactivity was electrophoresed on a 20% polyacrylamide gel containing 7 M urea as shown. The total (α-^{32}P)AMP incorporation at each dNTP concentration (per 25 µl reaction volume) was as follows: (1) 0 µM- 0.55 pmoles (100%), (2) 1.5 µM- 0.50 pmoles (93%), (3) 5 µM- 0.34 pmoles (63%), (4) 15 µM- 0.17 pmoles (31%), (5) 50 µM- 0.075 pmoles (14%).


1
2
3
4
5
– XyC
– BpB
0
1.5
5
15
50
dNTP conc., μM

synthesized in reactions containing 25 μM GTP, 5 μM ATP and 0, 1.5, 5, 15 or 50 μM dATP. The yield of total product, measured by (α-^{32}P)GMP incorporation, was nearly the same at each dATP concentration (Figure 23), indicating that dATP does not significantly inhibit primer synthesis on St-1 DNA in a non-competitive fashion. The products of each reaction were exclusively 11 nucleotides long as determined by polyacylamide gel electrophoresis (data not shown).

To determine the dAMP content of the hybrid primers, each product was digested to completion with ribonuclease T_1 and fractionated by electrophoresis at pH 1.9 on DEAE-81 paper as shown in Figure 23. Lanes (1) through (5) represent the resulting digest patterns of the primers synthesized in the presence of 0, 1.5, 5, 15 and 50 μM dATP. As expected, the oligoribonucleotide primer yields 5 ribonuclease T_1 digest products: pppApGp and ppApGp (positions 1,2), ApGp (positions 4,5), Gp (position 6) and ApApGp (positions 8-10). The molar ratio of radioactivity found in each digest nucleotide confirms that predicted for the transfer of label from (α-^{32}P)GMP (data not shown). If dATP has been incorporated into the hybrid primers at every position at which AMP is found in the oligoribonucleotide primer, the same treatment is expected to release four sets of hybrid digest nucleotides: ppp(A/dA)pGp, pp(A/dA)pGp, (A/dA)p(A/dA)pGp and (A/dA)pGp. Indeed, one of these sets, (A/dA)pGp, is resolved upon electrophoresis (Figure 23). Therefore, the preference for the utilization of ATP and dATP at position 4 may be determined from the fraction of radioactivity found in ApGp and (dA)pGp (Table 8).

The compositionally distinct nucleotides (A/dA)p(A/dA)pGp were not resolved by these electrophoretic conditions. Each hybrid trinucleotide

Figure 23 Contribution of ATP and dATP to primer synthesis on St-1 DNA

Reaction mixtures (20 µl) contained 4.3 nmoles of St-1 DNA, 1.3 units of dnaG protein, 2.8 µg of DNA binding protein, 25 µM (α-^{32}P)GTP, 5 µM ATP, and 0, 1.5, 5, 15 or 50 µM dATP as indicated. After 20 minutes at 30°C, mixtures were filtered through Sepharose 6B to remove unreacted substrate, digested with ribonuclease T_1 and electrophoresed at pH 1.9 on DEAE-81 paper (4,000 volt-hr) as shown. Lane (M) contained (^{32}p)ppApGp. The total (α-^{32}P)GMP incorporation at each dATP concentration was as follows: 0 µM- 3.1 pmoles; 1.5 µM- 2.6 pmoles; 5 µM- 2.6 pmoles; 15 µM- 2.5 pmoles; 50 µM- 2.3 pmoles. Preference for dAMP incorporation was determined as follows: position 4- relative proportion of ApGp and (dA)pGp; positions 8 and 9- analysis of (A/dA)p(A/dA)pGp as described in Figure 24; position 1- analysis of ppp(A/dA)pGp and pp(A/dA)pGp as described in Figures 25 and 26. Results are summarized in Table 8.

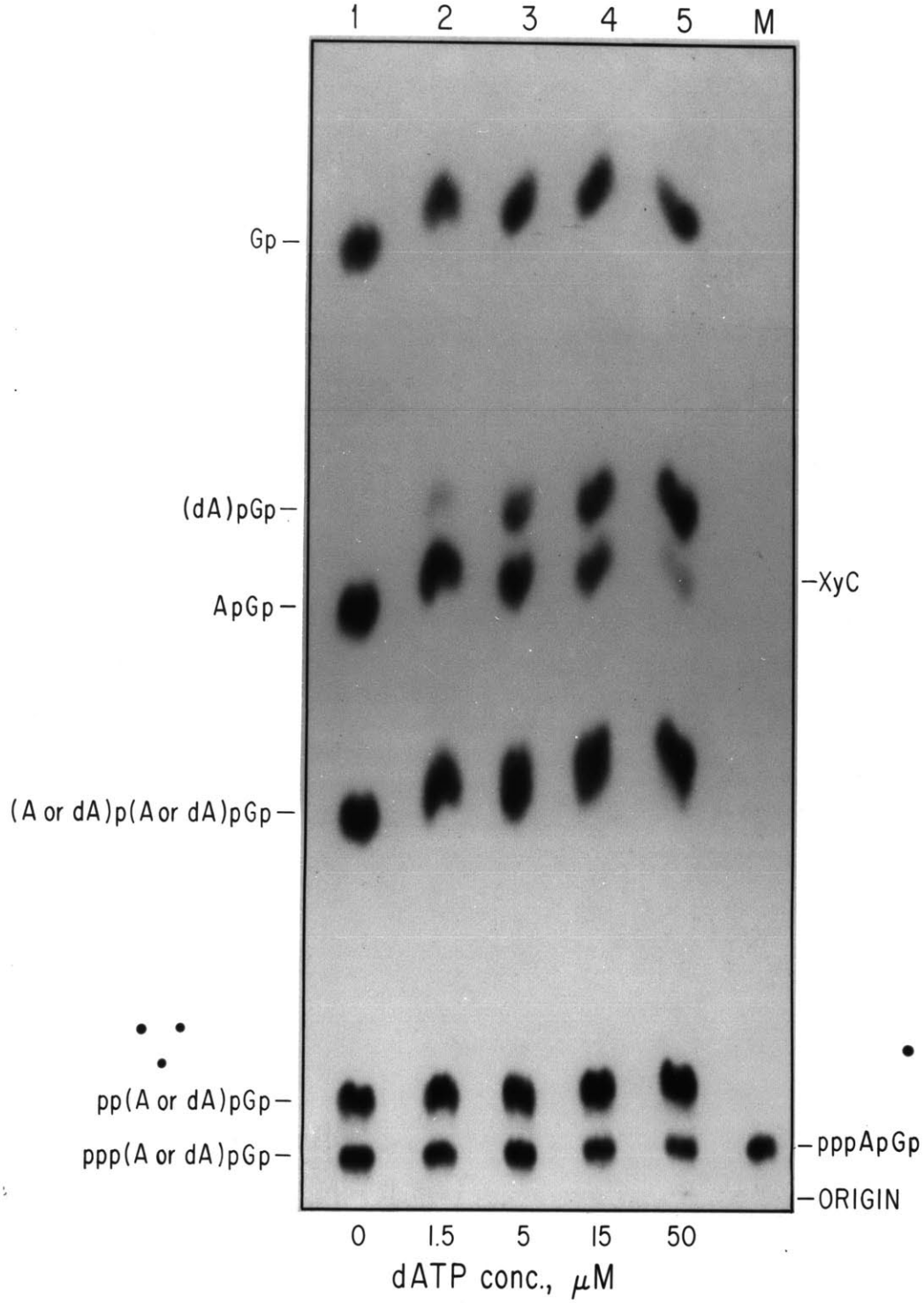

1
2
3
4
5
M
Gp
(dA)pGp
ApGp
(A or dA)p(A or dA)pGp
pp(A or dA)pGp
ppp(A or dA)pGp
XyC
pppApGp
ORIGIN
0
1.5
5
15
50
dATP conc., μM

Table 8 dATP is utilized efficiently as a substrate for primer synthesis on St-1 DNA

	Preference for dAMP incorporation				
Position	0 μM dATP	1.5 μM dATP	5 μM dATP	15 μM dATP	50 μM dATP
1- ppp(A/dA)pGp	0%	2.0%	3.4%	8.7%	28.9%
1- pp(A/dA)pGp	0	4.2	8.8	26.5	50.3
4	0	15.7	35.3	52.2	76.2
8	0	20.7	43.2	61.0	88.0
9	0	20.8	50.0	66.2	92.9

dATP utilization was determined for each position as described: position 1/ ppp(A/dA)pGp- Figure 25; position 1/pp(A/dA)pGp- Figure 26; position 4- Figure 23; positions 8 and 9- Figure 24.

was analyzed further by treatment with alkali to hydrolyze all 3' rNMP linkages followed by re-electrophoresis as shown in Figure 24. The trinucleotide released from the oligoribonucleotide primer is hydrolyzed completely to AMP and GMP (lane 1). In contrast, the trinucleotides released from the hybrid primers exhibit alkali-resistant linkages that indicate the incorporation of dAMP residues at position 8- (dA)pAp, at position 9- (dA)pGp, and at both positions- (dA)p(dA)pGp (lanes 2-5). The results of this analysis, taking into consideration the ratio of (^{32}P) transferred from neighboring GMP residues, provide enough information to calaculate the preference for utilization of dAMP at positions 8 and 9 according to the following relationships,

$$P_{dAMP}(8) = \frac{2\{(dA)pAp\} + \{(dA)p(dA)pGp\}}{\text{Total radioactivity}}$$

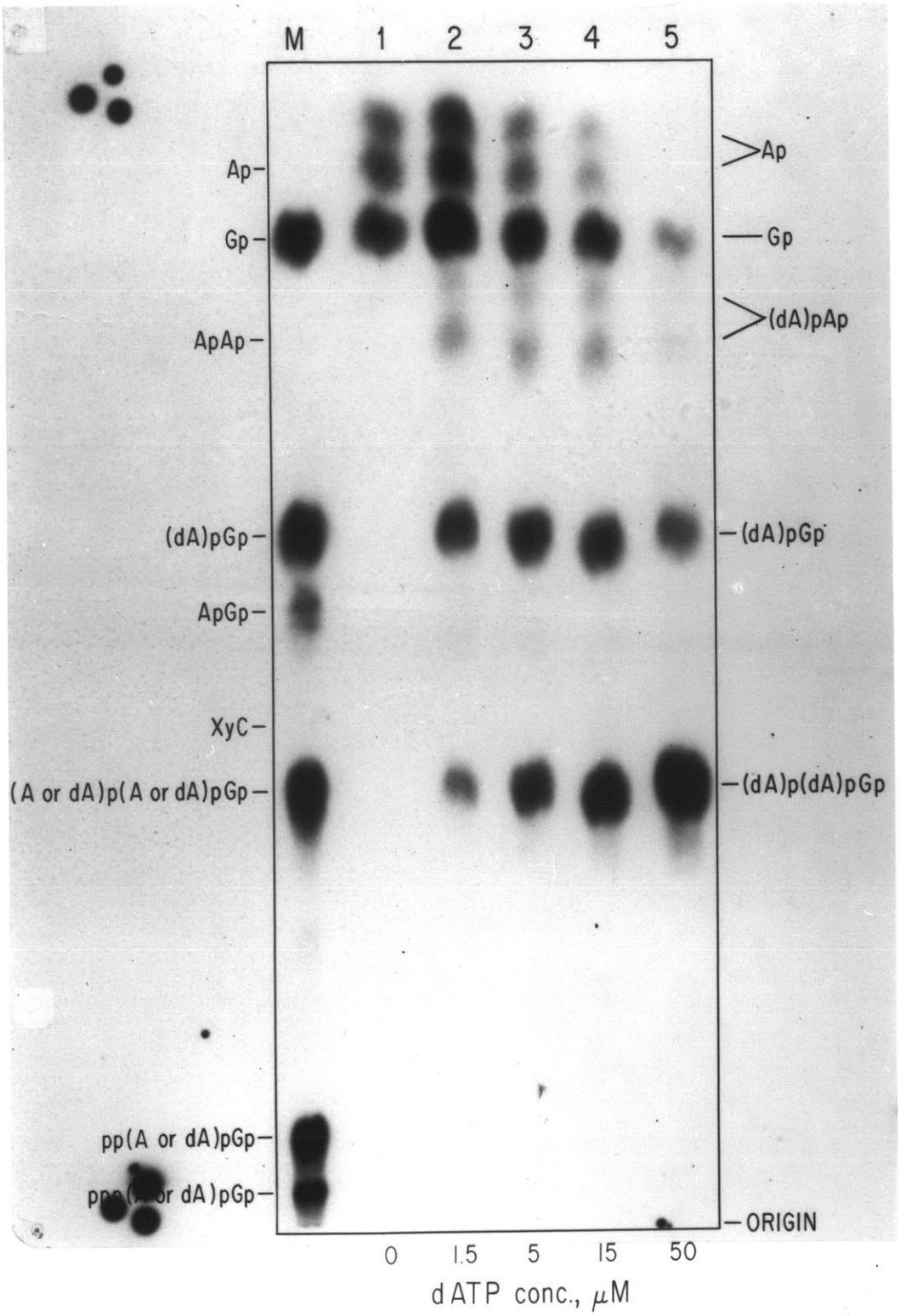
$$P_{dAMP}(9) = \frac{\{(dA)pGp\} + \{(dA)p(dA)pGp\}}{\text{Total radioactivity}}$$

where the quantities in brackets represent the amount of radioactivity found in the corresponding alkali-resistant nucleotides.

The results concerning the preference for dAMP at positions 4, 8 and 9 are summarized in Table 8. At each level of dATP examined, dAMP is incorporated to a similar extent at all three positions. Given the observation that the extent of total primer synthesis, measured by GMP incorporation, is unaffected by the addition of dATP to the reaction, it may be concluded that dATP and ATP are utilized with equal efficiency (35-50%) when each is present at the same initial concentration (5 μM).

Figure 24 Preference for dATP utilization at positions 8 and 9 of the St-1 primer

Each trinucleotide, (A/dA)p(A/dA)pGp, released by ribonuclease T_1 digestion of the hybrid primers in Figure 23 was recovered from the DEAE-81 paper, hydrolyzed with alkali and re-electrophoresed at pH 1.9 on DEAE-81 as shown. Lane (M) contained the entire ribonuclease T_1 digest of the hybrid primer synthesized at 50 μM dATP (Figure 23, lane 5), and 0.5 OD_{260} units each of 3'-AMP and ApAp. Preference for dAMP incorporation was calculated as described in the text and is summarized in Table 8.


M
1
2
3
4
5
Ap
Gp
ApAp
(dA)pGp
ApGp
XyC
(A or dA)p(A or dA)pGp
pp(A or dA)pGp
pp(A or dA)pGp
Ap
Gp
(dA)pAp
(dA)pGp
(dA)p(dA)pGp
ORIGIN
0
1.5
5
15
50
dATP conc., μM

In addition, these results demonstrate that AMP and dAMP are incorporated equally well onto a growing primer chain with a 3' ribonucleotide or deoxyribonucleotide terminus. For example, at 5 μM ATP and dATP, the preference for dAMP incorporation at position 9 is 45% and 57% when position 8 is occupied by an AMP and dAMP residue, respectively (data not shown). Thus, during primer elongation the dnaG protein selects dATP and ATP in proportion to the relative availability of each, with little preference shown in regard to the position on the primer chain or the composition of the preceding residue.

The contribution of dATP as the initiating nucleotide was determined by analyzing the dAMP content of the 5' terminal ribonuclease T_1 digest nucleotides, ppp(A/dA)pGp and pp(A/dA)pGp, which are separated according to charge (number of phosphate groups) but not on the basis of composition during electrophoresis at pH 1.9 on DEAE-81 paper (Figure 23). Each 5'-triphosphoryl terminated nucleotide was digested with alkali to split all 3' rAMP linkages and electrophoresed on a 20% polyacrylamide gel to reveal any alkali-resistant linkages as shown in Figure 25. As expected, the 5'-triphosphoryl nucleotide released from the oligoribonucleotide primer is completely hydrolyzed to pppAp and Gp (lane 1), which migrate faster and slower, respectively, than the undigested marker (^{32}p)ppApGp (lane M). In contrast, the 5'-triphosphoryl nucleotides released from the hybrid primers are partially resistant to alkali hydrolysis, indicating that dATP has been incorporated at the initiating position (lanes 2-5). Similar results are obtained when the 5'-diphosphoryl nucleotides are analyzed in the same fashion as shown in Figure 26.

The identity of the alkali-resistant 5'-triphosphoryl nucleotide

Figure 25 Preference for dATP utilization at the initiating position of St-1 primers with a 5'-triphosphoryl terminus

Each 5'-triphosphoryl nucleotide, ppp(A/dA)pGp, released by ribonuclease T_1 digestion of the hybrid primers in Figure 23 was recovered from the DEAE-81 paper, hydrolyzed with alkali and electrophoresed on a 20% polyacrylamide/7 M urea gel as shown. Lane (M) contained (^{32}p)ppApGp. The identity of the alkali-resistant nucleotide was confirmed as described in Figure 27. The preference for dATP incorporation at the initiating position was calculated from the fraction of alkali-resistant nucleotide and is summarized in Table 8.

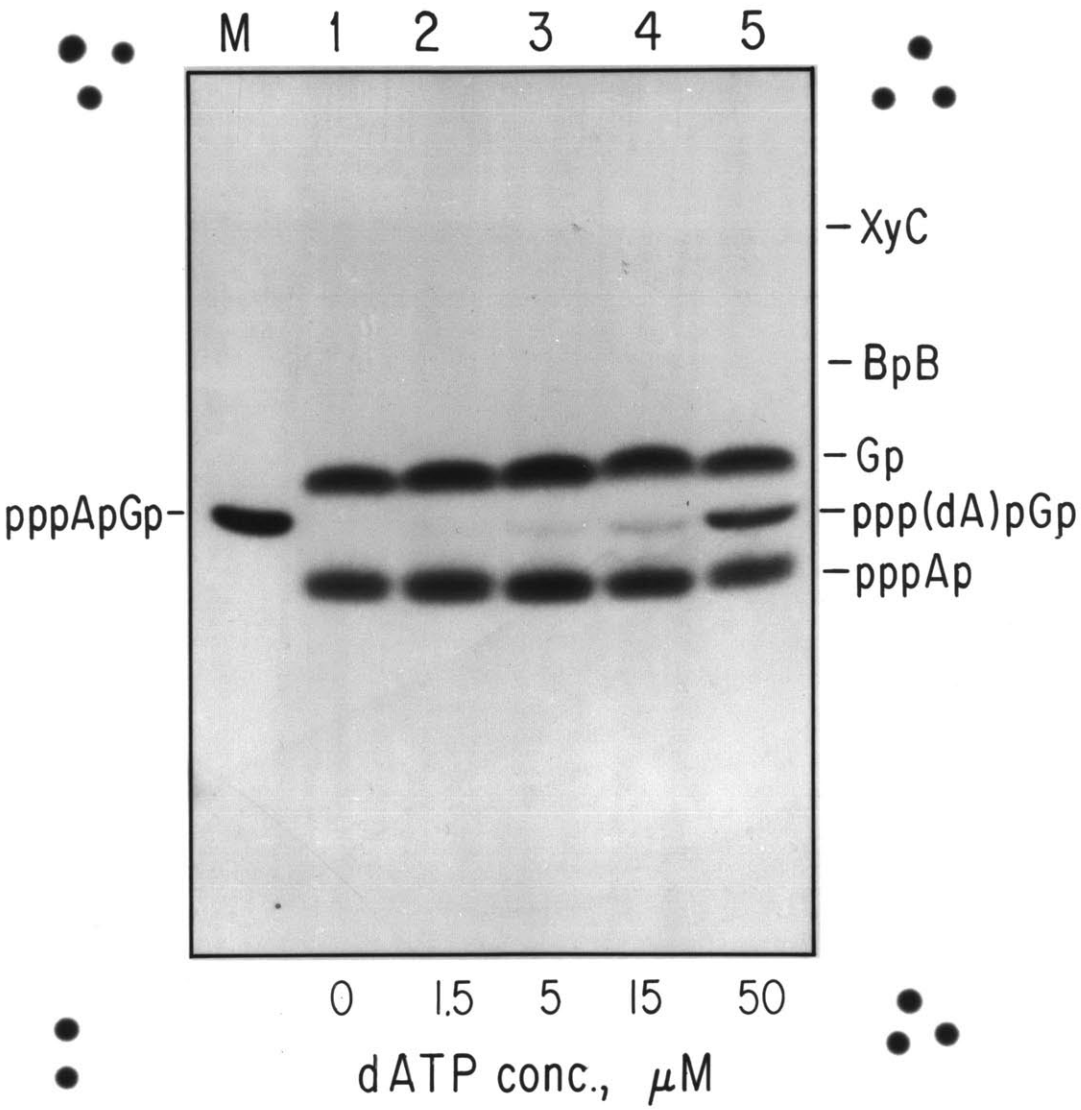
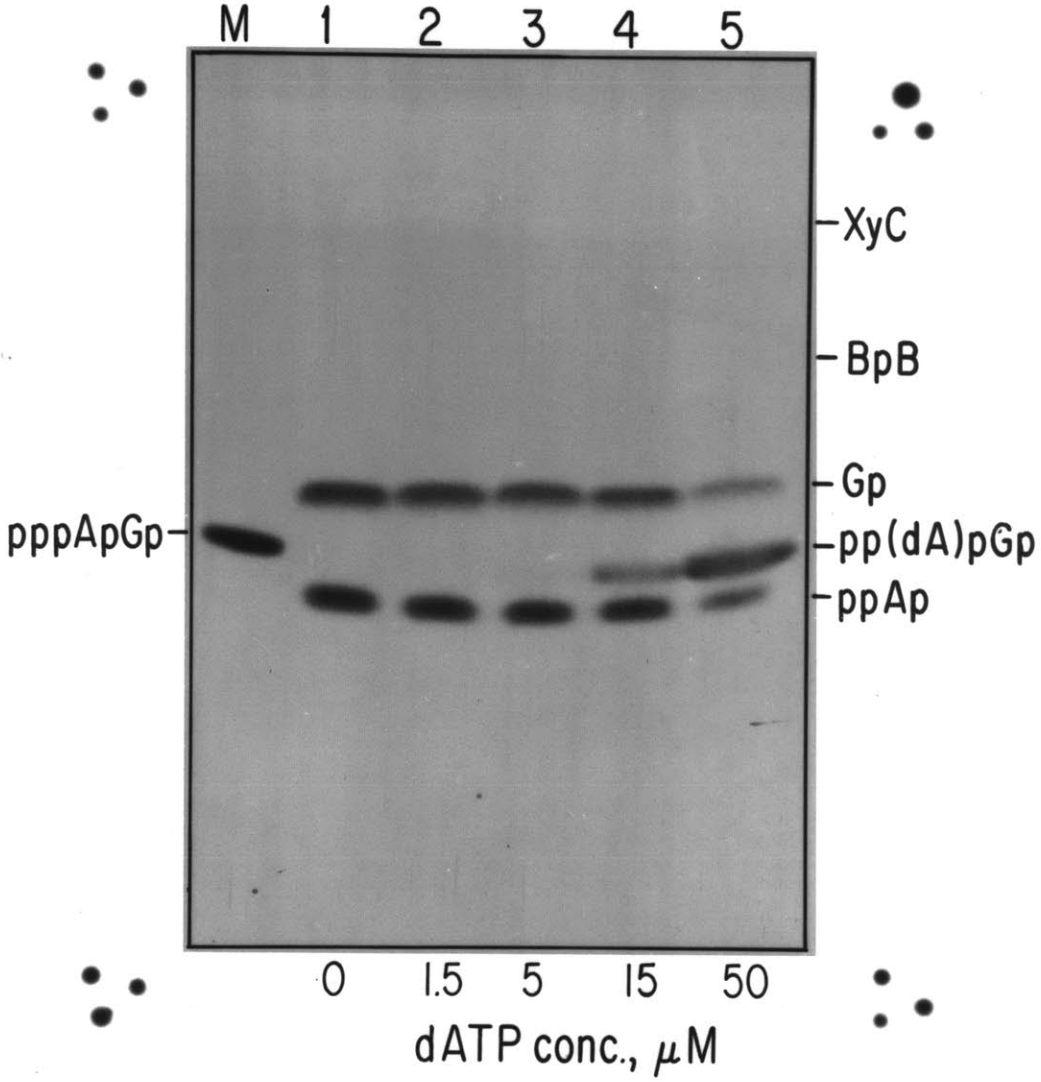

M
1
2
3
4
5
XyC
BpB
Gp
pppApGp
ppp(dA)pGp
pppAp
0
1.5
5
15
50
dATP conc., μM

Figure 26 Preference for dATP utilization at the initiating position of St-1 primers with a 5'-diphosphoryl terminus

Each 5'-diphosphoryl nucleotide, pp(A/dA)pGp, released by ribonuclease T_1 digestion of the hybrid primers in Figure 23 was analyzed as described in Figure 25. Results are summarized in Table 8.


M
1
2
3
4
5
XyC
BpB
Gp
pppApGp
pp(dA)pGp
ppAp
0
1.5
5
15
50
dATP conc., μM

was shown to be ppp(dA)pGp as follows. After recovery from the gel, the nucleotide was dephosphorylated with E. coli alkaline phosphatase, and digested with calf spleen phosphodiesterase. This treatment is expected to release either dAMP or AMP, which may be separated by chromatography on PEI-cellulose (Randerath, 1967), depending on whether primer synthesis has been initiated with dATP or ATP. As seen in Figure 27, the alkali-resistant 5'-triphosphoryl nucleotide indeed yields dAMP (lane 6), whereas pppApGp yields AMP (lane 7).

The preference for dATP incorporation at the first position is summarized in Table 8. It is evident that dATP is utilized less efficiently for primer initiation than elongation. However, other experiments suggest that primer chains are initiated effectively with dATP in the absence of ATP. When synthesis is performed in the presence of the four dNTPs only, 1 pmole of St-1 DNA molecules supports the incorporation of 1.7 pmoles of (α-^{32}P)dATP into primers 20-25 nucleotides long that are resistant to alkali (data not shown). This is comparable to primer synthesis in the presence of rNTPs (Table 6).

A different approach was used to determine the positional preference for dGTP and GTP (Figure 28). 5' terminally labeled primers were synthesized in reactions containing (γ-^{32}P)ATP and either 25 µM GTP (lane 1) or 25 µM each GTP and dGTP (lane 3). Each product is 11 nucleotides long. The oligoribonucleotide primer was partially hydrolyzed with alkali to provide chain length markers of 1 to 11 nucleotides in length (lane 5). Exhaustive digestion of the hybrid primer with ribonuclease T_1 produces a set of radioactive fragments that extend from the labeled 5' end to each position at which GMP is known to occur from the St-1 primer sequence

Figure 27 Primer synthesis on St-1 DNA is initiated by dATP or ATP

Lanes (6) and (7) represent the alkali-resistant nucleotide, ppp(dA)pGp (Figure 25, lane 5), and pppApGp (Figure 23, lane 1) analyzed as follows. Each nucleotide was dephosphorylated with E. coli alkaline phosphatase, digested with calf spleen phosphodiesterase and chromatographed on a PEI-cellulose thin-layer as described in Methods. Lane (M) contained $^{32}P_i$, and 0.5 OD_{260} units each 3'-dAMP and 3'-AMP. Lanes (1) through (5) show the same analysis performed on each 5'-triphosphoryl nucleotide released by ribonuclease T_1 digestion of the hybrid primers in Figure 23.

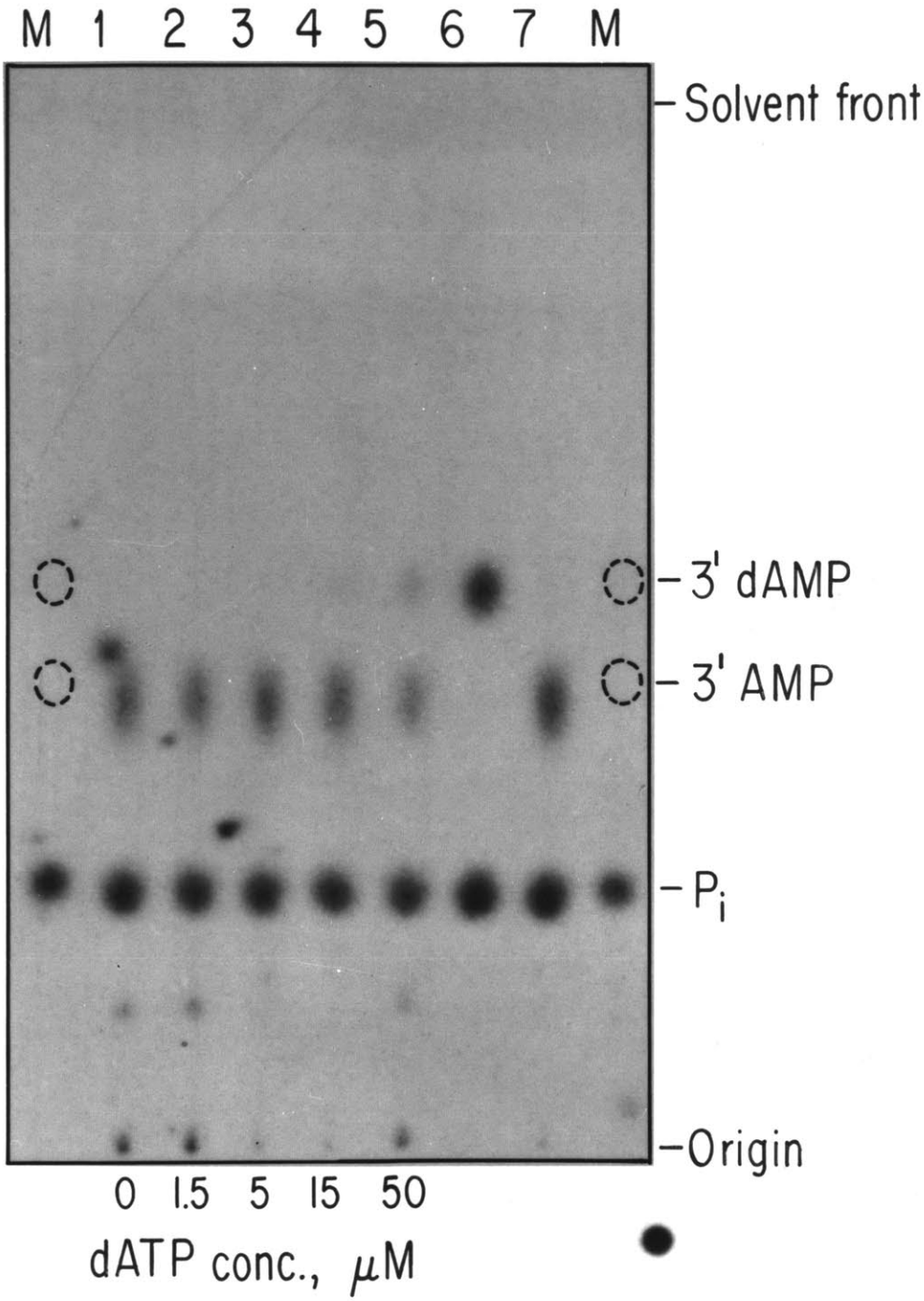
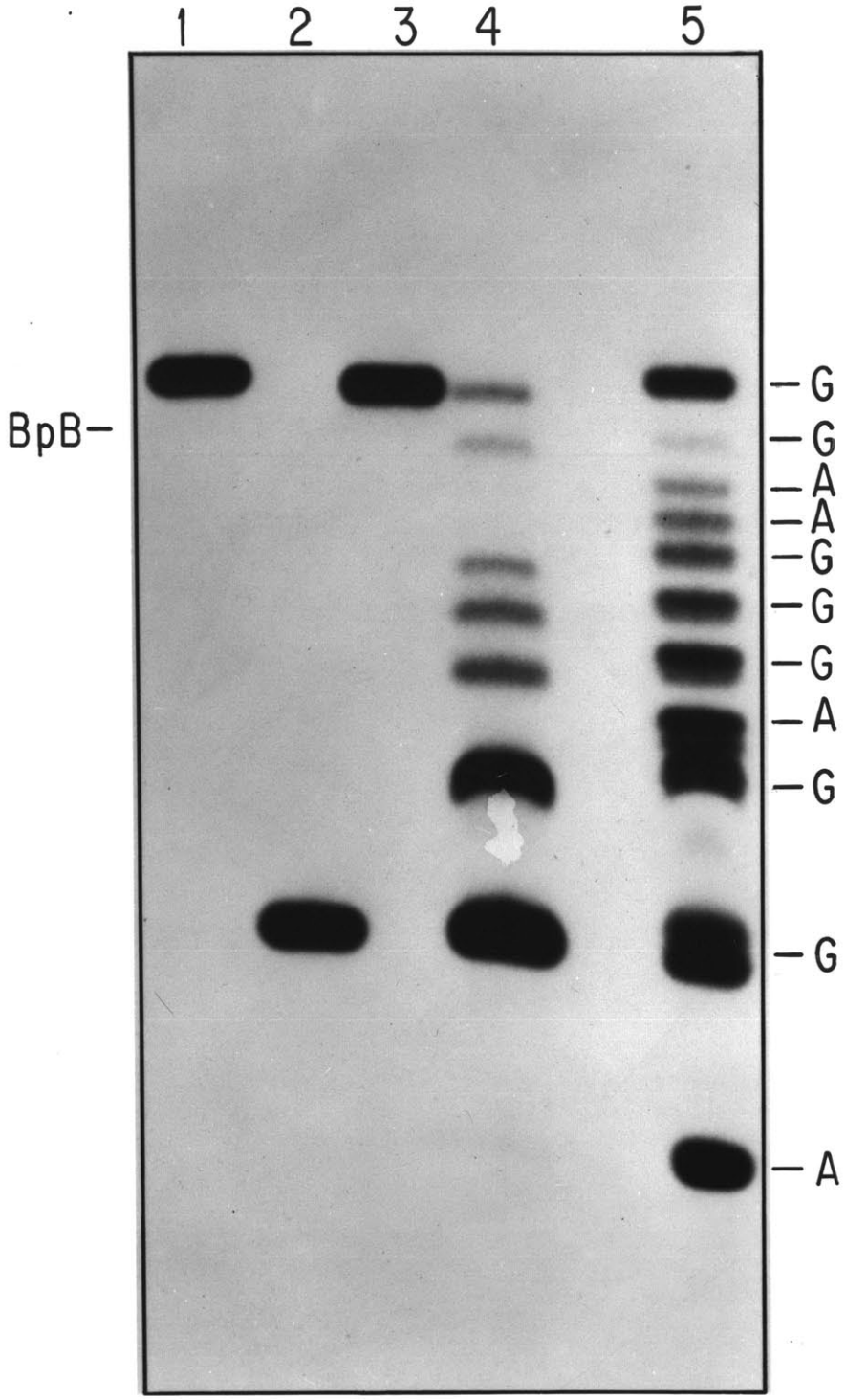

M 1 2 3 4 5 6 7 M
Solvent front
3' dAMP
3' AMP
P_i
Origin
0 1.5 5 15 50
dATP conc., μM

Figure 28 Contribution of GTP and dGTP to primer synthesis on St-1 DNA

Reaction mixtures (20 µl) contained 3.0 nmoles of St-1 DNA, 1.2 units of dnaG protein, 1.9 µg of DNA binding protein, 20 µM (γ-^{32}P)ATP (2900 Ci/mmole) and either 25 µM GTP (lane 1) or 25 µM each GTP and dGTP (lane 3). After 20 minutes at 30°C, each mixture was filtered through Sepharose 6B to remove unreacted substrate, and an aliquot was electrophoresed on a 20% polyacrylamide/7 M urea gel as shown. Lane (5) represents a partial alkali digest of the oligoribonucleotide product. Lanes (2) and (4) show a ribonuclease T_1 digest of the oligoribonucleotide and hybrid products, respectively. Each band in lane (4) was cut out and the radioactivity was determined by liquid scintillation counting (Table 9). Incorporation of (γ-^{32}P)ATP was 0.12 pmoles and 0.081 pmoles in the absence and presence of dGTP, respectively.


1
2
3
4
5
BpB–
– G
– G
– A
– A
– G
– G
– G
– A
– G
– G
– A

(lane 4). Under the same digest conditions, the oligoribonucleotide primer is completely degraded to the 5' end-labeled dinucleotide pppApGp (lane 2). Since the fragments resulting from the digestion of the hybrid primer are resistant to further cleavage by ribonuclease T_1 at all internal positions, it is concluded that dGMP may be incorporated at every position at which a GMP residue is otherwise found.

Assuming that each fragment cleaved at a specific position represents those primers which have GMP incorporated at that site, whereas primers which have dGMP at the same position are represented by longer cleavage products, the preference for dGMP incorporation at position N may be calculated as follows,

$$P_{dGMP}(N) = 1 - \frac{\text{Fragment}(N)}{\sum_{i \geq N} \text{Fragment}(i)}$$

where Fragment(N) represents the amount of radioactivity found in a ribonuclease T_1 fragment of length N, measured directly in the experiment shown in Figure 28. The results of this calculation for dGMP preference at positions 2, 3, 5, 6, 7 and 10 are summarized in Table 9. A preference for position 11 cannot be determined since the fragment of this length may have either a GMP or dGMP residue at its 3' terminus. dGTP is incorporated with a similar efficiency at each position in the primer. Overall, GTP and dGTP contribute about equally to primer elongation when each is available at the same concentration (25 μM).

Table 9 dGTP is utilized efficiently as a substrate for primer synthesis on St-1 DNA

Position (N)	Amount of radioactivity found in T1 fragment of length N Fragment(N)[1]	Fraction of primers with dGMP residue incorporated at position N $P_{dGMP}(N)$[2]
2	24,710 cpm	40%
3	12,330	24
5	1,350	66
6	980	62
7	560	65
10	310	70
11	740	-

[1] Determined from the experimental results shown in Figure 28

[2] Calculated from the following equation,

$$P_{dGMP}(N) = 1 - \frac{\text{Fragment}(N)}{\sum_{i \geq N} \text{Fragment}(i)}$$

The nature of the primer that serves to initiate synthesis of the complementary strand on St-1 DNA

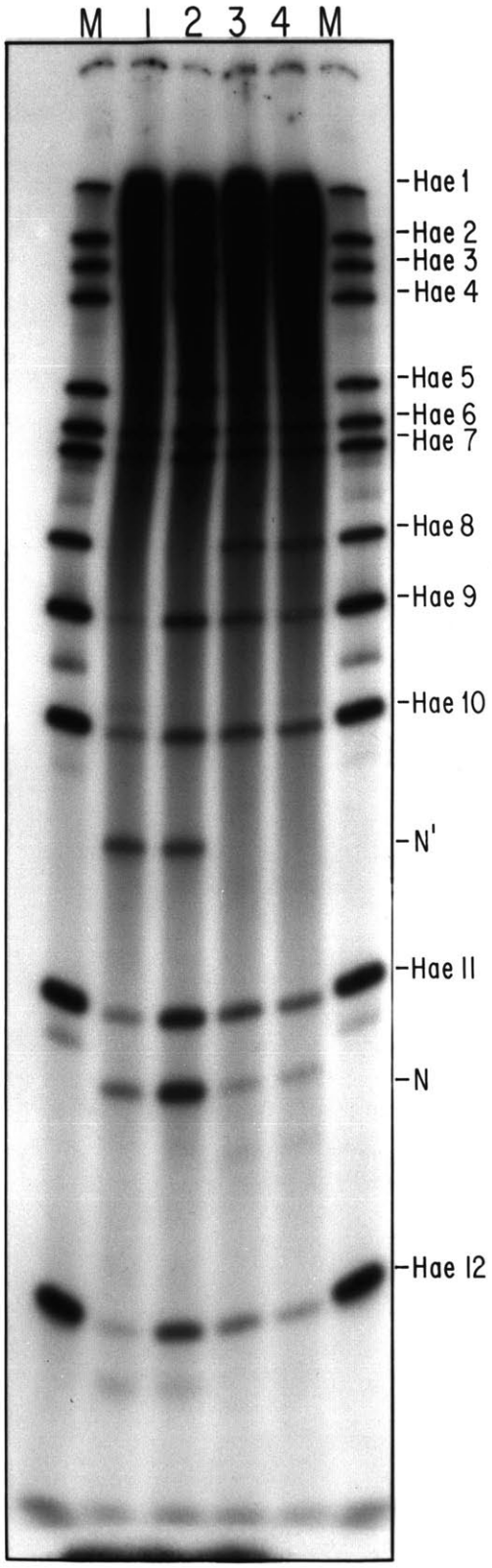
The above description of primer synthesis pertains to the reaction catalyzed by the dnaG protein in the absence of DNA synthesis. To determine the nature of primer formation when coupled to DNA synthesis, the 5' end of the nascent DNA product synthesized on St-1 single-stranded DNA was analyzed. The assumption made in these experiments is that the occurence of a ribonucleotide at a specific location in the DNA product will indicate that priming by the dnaG protein has proceeded as far as that position.

Since the outcome of coupling the priming step to DNA synthesis would possibly be influenced by the proteins employed to catalyze DNA elongation, the effect of including in the reaction either DNA polymerase III with the dnaZ protein and elongation factors I and III, or DNA polymerase I, was investigated. In each case, two reactions were compared. The first contained all of the necessary components- St-1 DNA, DNA binding protein, DNA polymerase and elongation factors, rNTPs and dNTPs- and was initiated by the addition of the dnaG protein to ensure that DNA synthesis would be maximally coupled to the priming event. In the second reaction, priming was allowed to proceed to completion before the addition of DNA polymerase and elongation factors. This is referred to as the uncoupled reaction.

The products of each reaction were first digested with restriction endonuclease Hae III and the digest was then fractionated on a polyacrylamide gel in order to isolate a short DNA segment of defined length containing the 5' end of the nascent DNA strand (Figure 29). Lanes (1) and (2) show the products of the coupled and uncoupled reactions that employed

Figure 29 Isolation of a restriction fragment containing the 5' end of the nascent complementary strand synthesized on St-1 DNA

Reaction mixtures (40 µl) contained reaction buffer (Table 5), 1.1 nmoles of St-1 DNA, µg of DNA binding protein, units of dnaG protein, 0.25 mM ATP, 0.05 mM each GTP, CTP and UTP, 0.05 mM each (α-^{32}P)dATP, dGTP, dCTP and TTP (200-350 Ci/mmole), and either units of DNA polymerase III, units of dnaZ protein, units of elongation factor I and units of elongation factor III (lanes 1,2), or units of DNA polymerase I (lanes 3,4). In lanes 1 and 3, the priming and DNA elongation reactions were fully coupled by mixing all of the components at once and initiating the reaction by the addition of the dnaG protein. In lanes 2 and 4, the priming step was uncoupled from DNA elongation by first incubating the DNA, DNA binding protein, dnaG protein, rNTPs and dNTPs for 15 minutes at 30°C, and then adding DNA polymerase and elongation factors. Once DNA polymerase was added, each reaction was incubated for 20 minutes at 30°C, stopped by the addition of 0.05 M EDTA, filtered through Sepharose 6B to remove unreacted substrate, extracted with phenol and precipitated with ethanol (see Methods). Each product was digested with Hae III, denatured in 100% formamide and electrophoresed on a 4% polyacrylamide/7 M urea gel as shown. Lanes (M) contained a Hae III digest of St-1 RF I that was radioactively labeled with phage T4 polynucleotide kinase as described in Methods.


M
1
2
3
4
M
Hae 1
Hae 2
Hae 3
Hae 4
Hae 5
Hae 6
Hae 7
Hae 8
Hae 9
Hae 10
N'
Hae 11
N
Hae 12

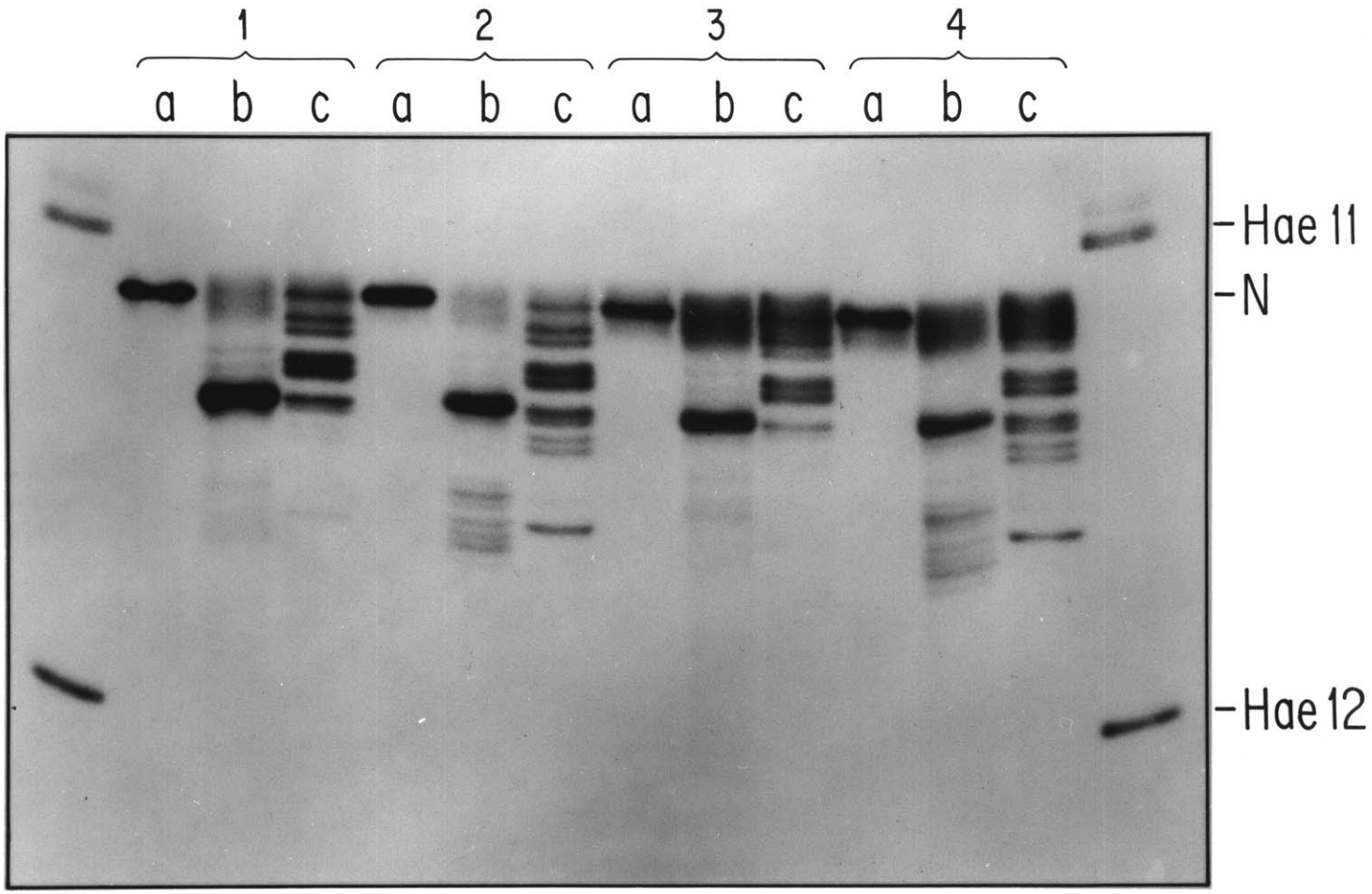
DNA polymerase III. Lanes (3) and (4) represent the coupled and uncoupled reactions with DNA polymerase I. The digest products were identified by comparison with the fragments produced by a Hae III digest of fully duplex St-1 RF I DNA (lane M). Each of the four reaction products releases a fragment, labeled N, which does not correspond to any of the fragments observed in the marker lane. N has been previously characterized as the 5' portion of the nascent DNA product, 105 nucleotides long, that extends from the origin of complementary strand synthesis on St-1 DNA to the first downstream Hae III site (Sims, Capon and Dressler, 1979).

Fragment N was analyzed for the presence of ribonucleotides at its 5' end according to the following rationale. Priming by the dnaG protein, and therefore incorporation of ribonucleotides, was expected to proceed no further than 25-30 nucleotides from the 5' terminus of the 105 nucleotide long fragment. Consequently, the reduction in size observed when the fragment is treated with alkali or ribonuclease will reveal the furthest extent along the potential primer sequence that a ribonucleotide had been incorporated. The original product had been synthesized in reactions containing (α-^{32}P)dNTPs; therefore, fragment N is effectively labeled at its 3' end for the purpose of this analysis.

Fragment N, prepared from each of the four reaction products, was treated with alkali or ribonuclease to hydrolyze all susceptible 3' rNTP linkages and electrophoresed on a polyacrylamide gel as shown in Figure 30. Lanes (2a) through (2c) represent the analysis of the 5' end of products synthesized in the uncoupled reaction containing DNA polymerase III. The untreated fragment is homogeneous in size (lane 2a), confirming that primer formation begins at a unique site. Digestion with ribonuclease T_1 produces

Figure 30 Length of the primer that initiates synthesis of the complementary strand on St-1 DNA.

Fragment N was prepared as described in Figure 29: Lanes (1)- coupled, DNA polymerase III; Lanes (2)- uncoupled, DNA polymerase III; Lanes (3)- coupled, DNA polymerase I; Lanes (4)- uncoupled, DNA polymerase I. Each fragment was recovered from the gel (see Methods) and electrophoresed on a 10% polyacrylamide/7 M urea gel after the following treatment: Lanes (a)- not treated; Lanes (b)- alkali hydrolysis; Lanes (c)- ribonuclease T_1 digestion. The marker lane contained a Hae III digest of St-1 RF I DNA (see Figure 29). Hae 11 and Hae 12 are approximately 115 and 70 bases long, respectively.


1
2
3
4
a b c a b c a b c a b c
Hae 11
N
Hae 12

10 additional discrete fragments that range from 85 to 103 nucleotides long (lane 2c). The length observed for each digest product is consistent with that expected if cleavage occurred at each of the 10 positions known from the sequence of the St-1 primer to contain a GMP or dGMP residue (compare with Figure 11). Each species represents the proportion of the DNA product in which priming by the dnaG protein had proceeded at least as far as the position at which the GMP residue is detected. When the same product is hydrolyzed with alkali, most of the digest products are approximately 95 nucleotides long (lane 2b). This demonstrates that greater than 90% of the DNA product was initiated from a primer that is at least 9-11 nucleotides in length. A smaller proportion of the DNA product was extended from longer primers that range up to 23 nucleotides. As expected, the same results are observed for the uncoupled reaction that contained DNA polymerase I (lanes 4a-c).

Lanes (1a) through (1c) show the analysis of the 5' end of products synthesized by DNA polymerase III when priming and DNA synthesis are coupled. It is clearly evident that a similar large proportion of the DNA product is initiated from primers 9-11 nucleotides long. However, in contrast to the DNA product synthesized in the uncoupled reactions, substantially less product is initiated from longer primers. The extent of coupling observed with DNA polymerase I (lanes 3a-c) is very similar to that found with DNA polymerase III. Thus, although primer formation is interrupted when coupled to DNA synthesis, the effect is not specific for either DNA polymerase.

As seen in Figure 29, in addition to N there is a second fragment, labeled N', which does not correspond to any of the fragments produced by

Hae III digestion of St-1 RF DNA. Fragment N' appears in the digests of the products synthesized by DNA polymerase III (lanes 1,2), but not those synthesized by DNA polymerase I (lanes 3,4). When analyzed in the fashion described above, N' was shown not to contain any ribonucleotide residues (data not shown). The length of N', 150 bases, is consistent with that expected for the Hae III digest product containing the 3' portion of the nascent DNA product. If this is correct, then N' and N together comprise the origin-containing fragment, Hae 8. Consistent with this, the absence of N' in digests of the DNA polymerase I products is compensated by the presence of a fragment corresponding to Hae 8 (Figure 29). The latter fragment does not occur in the digest of the DNA polymerase III products, and presumably arises because DNA polymerase I is able to catalyze DNA synthesis through the origin-containing region by a strand displacement mechanism.

Discussion

This study has shown that the dnaG protein initiates the synthesis of the complementary strand on St-1, øK and α3 single-stranded phage DNA in the presence of the DNA binding protein by catalyzing the synthesis of a specific oligoribodeoxyribonucleotide primer. Similar investigations have shown that the dnaG protein may utilize either rNTPs (Bouche, Zechel and Kornberg, 1975) or rNTPs and dNTPs (Wickner, 1977, Rowen and Kornberg, 1978b, Capon and Gefter, 1978) to synthesize a primer on phage G4 DNA. In contrast to the latter studies, the results presented here demonstrate that the dnaG protein may efficiently synthesize primers on St-1 DNA with dNTPs alone (see below).

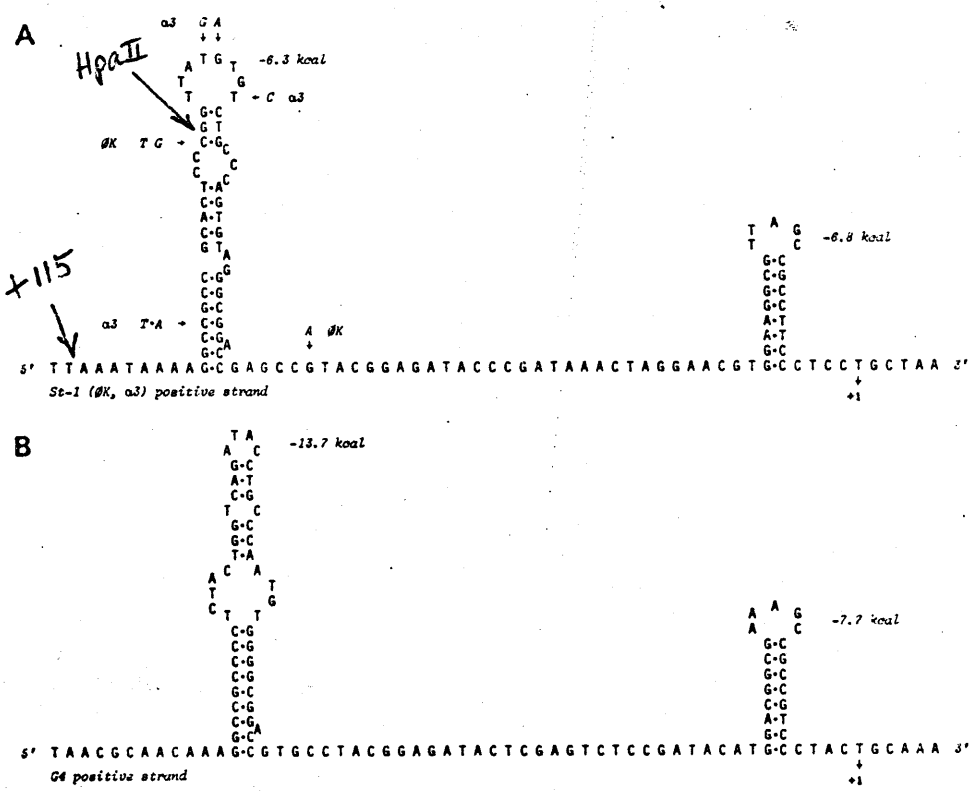
Together with the reported sequence for the G4 ribonucleotide primer (Bouche, Rowen and Kornberg, 1978, D. Capon and S. Wickner, unpublished observations), the sequencing studies described here demonstrate that the specificity of this priming pathway, which does not operate on other DNAs including øx174 or fd phage DNA, consists in the transcription of a unique and highly conserved sequence on these phage templates. Primer formation on St-1 (øK, α3) DNA is initiated with ATP or dATP (see below) at a unique site as determined by (1) the 5' terminal sequence of the oligonucleotide primer, and (2) the singular length of the Hae III digest fragment that contains the 5' terminal portion of the nascent DNA product (Figure 30). Primer synthesis extends for 10-28 nucleotides, copying a sequence that is identical on St-1, øK and α3 DNA, and that differs by 5 nucleotides on G4 DNA. This sequence lies in an intercistronic region of approximately 135 nucleotides, containing two highly conserved 40-45 base stretches that

are separated by 13 bases of divergent sequence (Sims and Dressler, 1978, Fiddes, Barrell and Godson, 1978, Sims, Capon and Dressler, 1979). Each conserved region exhibits the capacity to form secondary structure: the hairpin at positions +6 to +26 that is transcribed by the dnaG protein, and a larger hairpin further downstream at positions +62 to +107 (Figure 31). It is therefore possible that the dnaG protein binds specifically to the template by recognizing either a conserved nucleotide sequence, such as a promoter for RNA polymerase, and/or secondary structure. The following observation support a functional role for the latter: (1) The two base differences between the St-1 (øK, α3) and G4 'primer' hairpins that occur on the stem compensate to preserve base-pairing (Figure 11). In contrast, the respective loops are different at 2 out of 5 positions. (2) Evidence for the stability of the primer hairpin is indicated by the preference for ribonuclease T_1 cleavage of the corresponding oligoribonucleotide primer at the proposed single-stranded rather than base-paired positions. (3) The length of the primer appears to be determined by conditions that affect the stability of secondary structure. Less primer is elongated beyond positions 10-12 at lower temperatures and higher salt concentrations.

The role of the DNA binding protein in promoting the action of the dnaG protein on St-1, øK, α3 and G4 DNA may be related to the protein's ability to destabilize secondary structure (Sigal et al., 1972). It has been shown here that dnaG-dependent DNA synthesis on St-1 DNA exhibits a higher-order dependence upon the concentration of DNA binding protein. A similar observation has been made concerning the DNA binding protein requirement for the binding of the dnaG protein to G4 DNA (Wickner, 1977).

Figure 31 Potential secondary structure surrounding the origin of complementary strand synthesis on St-1, øK, α3 and G4 phage DNA

Panel (A)- St-1, øK and α3 DNA; panel (B)- G4 DNA (taken from Sims, Capon and Dressler, 1979, Figure 14). The origins are at the positions marked +1. The 'primer' hairpin is on the right and the 'downstream' hairpin is on the left. The arrow indicates the site of cleavage by Hpa II on St-1 DNA.


A
HpaII
-6.3 kcal
-6.8 kcal
+115
5' TTAAATAAAAGCGAGCCGTACGGAGATACCCGATAAACTAGGAACGTGCCTCCTGCTAA 3'
St-1 (ØK, α3) positive strand
+1
B
-13.7 kcal
-7.7 kcal
5' TAACGCAACAAAGCGTGCCTACGGAGATACTCGAGTCTCCGATACATGCCTACTGCAAA 3'
G4 positive strand
+1

Since the DNA binding protein is known to bind to single-stranded DNA in a highly cooperative fashion (Sigal et al., 1972, Molineux, Pauli and Gefter, 1975), these results suggest that several or many molecules of DNA binding protein are required to bind to a molecule of DNA to create a functional substrate for priming. Such an interaction might lead to the formation of a high affinity binding site for the dnaG protein at the origin. Alternatively, the DNA binding protein may direct the dnaG protein to a specific site by preventing non-specific binding to other regions of the template, or through a protein-protein interaction as has been demonstrated between the DNA binding protein and E. coli DNA polymerase II or exonuclease I (Molineux and Gefter, 1974, 1975). Thus far, the experimental data do not rule out any of these possibilities.

In contrast to the site-specific mechanism of priming observed on St-1, øK, α3 and G4 DNA in the presence of the DNA binding protein, the dnaG and dnaB proteins together appear to synthesize primers at many distinct sites on øx174, M13 and G4 single-stranded DNA and on poly(dT) in the absence of the DNA binding protein (Arai and Kornberg, 1979). These findings suggest that the dnaB protein is capable of interacting with many different DNA sequences to produce a higher-order 'structure' similar to that recognized by the dnaG protein on St-1 DNA in the presence of DNA binding protein. Recently, it has been reported that the dnaG protein protects three well-separated groups of nucleotides on øK single-stranded origin DNA from cleavage by various nucleases: in and around the stem of the primer hairpin, and 7-8 nucleotides on either side of the 'downstream' hairpin (see Figure 31) (Sims and Benz, 1980). On the basis of studies that had showed that the dnaG protein acts with a stoichiometry

of one molecule per template (Wickner, 1977, Rowen and Kornberg, 1978a, Benz et al., 1979), the ability of the dnaG protein to protect distant groups of nucleotides has been interpreted as evidence that the protein recognizes a higher-order tertiary structure on the DNA. Current studies in this laboratory, however, indicate that the downstream hairpin as well as the most distal group of protected bases (positions +109 to +115) are dispensible to priming by the dnaG protein on St-1 DNA (D. Capon, unpublished observations). A 176 base long, single-stranded Hpa II fragment containing the St-1 origin (positions -84 to +92), that cuts through the middle of the downstream hairpin (see Figure 31), supports efficient synthesis of oligoribonucleotides, 9-12 bases long (data not shown). Studies to further delimit the region(s) on the template that function as 'origin', are in progress.

The most surprising aspect of dnaG protein function is the ability to polymerize both ribo- and deoxyribonucleotides. The reason for this capability is not clear at present. The experiments described here do rule against the possibility that dNTPs cause the termination of primer chains as previously suggested (Rowen and Kornberg, 1978b). First, dNTPs are as effective as rNTPs in overcoming the barrier to primer elongation at positions 10-12. Secondly, both dATP and dGTP substitute for the incorporation of the homologous rNTP at every position in the primer. When present at equal concentrations, rNTPs and dNTPs are utilized with the same efficiency for primer elongation. rNTPs and dNTPs are as likely to be incorporated after a ribo- or deoxyribonucleotide residue, or at any position in the primer. dATP as well as ATP is utilized effectively for primer initiation, although when the latter nucleotide is available,

there is a distinct preference for its incorporation. In contrast, it has been reported that dATP does not serve as the initiating nucleotide on G4 DNA (Rowen and Kornberg, 1978b). This difference may be related to the observation made here that ATP stimulates dnaG-dependent DNA synthesis on G4 DNA by 5-10 fold but is essentially not required with St-1 DNA. Indeed, this striking contrast in substrate utilization by the dnaG protein on templates with nearly identical nucleotide sequences suggests that other aspects of template structure affect the catalytic action of the enzyme as well as its recognition properties.

Primers initiated with dATP or ATP may have either a di- or triphosphoryl 5' terminus. The diphosphate end may result from degradation of the product or arise from the direct incorporation of dADP and ADP. The latter alternative is supported by the observation that the addition of ADP to reactions containing the four dNTPs leads to the synthesis of primers that are no longer alkali-resistant (data not shown). Another group has observed the incorporation of (β-^{32})ADP into primers on α3 DNA by preparations of dnaG protein free of nucleoside diphosphate kinase activity (Benz et al., 1979).

When primer formation is coupled to DNA synthesis, greater than 90% of the DNA product is initiated from primers that are 9-11 nucleotides long. The method employed to analyze the 5' end of the nascent DNA products would have quantitatively detected ribonucleotide primers as short as a mono- or dinucleotide. In contrast to these findings, it has been reported that primers as short as a dinucleotide are effective in initiating DNA synthesis on G4 DNA (Rowen and Kornberg, 1978b). This conclusion was based on the observation that in a ribonuclease T_1 digest of the

nascent DNA product synthesized in the presence of 20 μM rNTPs and 50 μM dNTPs, the initiating dinucleotide pppApGp was considerably more abundant than digest nucleotides representing other primer positions. However, this interpretation may not be correct. It has been demonstrated here that dNTPs are efficiently incorporated at all primer positions, and in fact, are expected to predominate when available in 2.5-fold excess over rNTPs. As a result, the incorporation of dGMP residues would be expected to preferentially render internal ribonuclease T_1 digest fragments resistant to cleavage.

Primer formation terminates earlier when coupled to DNA synthesis. This may be due to a physical coupling between priming and DNA elongation or simply the dissociation of the dnaG protein from the template during a rate-limiting step in primer synthesis, such as elongation past the barrier at positions 10-12. The latter is consistent with the finding that primer synthesis is affected similarly when coupled to DNA elongation by either DNA polymerase III with the dnaZ protein and elongation factors I and III or DNA polymerase I.

The dnaG protein is probably the enzyme that initiates DNA chains in E. coli. At present, however, the nature of this physiological event is not understood. One approach to determining the contribution of RNA and DNA priming to cellular DNA replication would be to isolate and analyze the 5' terminus of defined nascent DNA chains initiated by a similar mechanism, such as those corresponding to the complementary strand of phages St-1, øK, α3 and G4. Studies of this kind are currently in progress.

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